ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ ΙΑΤΡΙΚΗ ΣΧΟΛΗ

Διδακτορική Διατριβή

Ρύθμιση της ανοσοαπάντησης στις Ιδιοπαθείς Φλεγμονώδεις Νόσους του Εντέρου και σε πειραματικά μοντέλα κολίτιδας

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Πανεπιστήμιο Κρήτης Ιατρική Σχολή

Ηράκλειο, 2018

UNIVERSITY OF CRETE SCHOOL OF MEDICINE

PhD Thesis

Regulation of immune response in Inflammatory Bowel Disease and experimental colitis models

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Heraklion, 2018

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1. ABSTRACT

Inflammatory bowel disease (IBD) is a chronic remittent or progressive inflammation of the gastrointestinal tract that affects millions of people worldwide. This inflammatory condition encompasses two major forms known as Crohn's disease (CD) and ulcerative colitis (UC). The onset of IBD typically occurs in the second and third decades of life, with the majority of patients progressing to a relapsing and chronic disease. Although the precise cause of IBD has not yet been fully elucidated, recent advances in the understanding of the molecular pathogenesis of IBD have been made owning to three related lines of investigation. First, IBD has been amenable to the discovery of susceptibility genes. Secondly, it appears that commensal pathogens or their products rather than conventional flora, play a pivotal role in the dysregulated immunity typically observed in IBD cases. Thirdly, murine models that exhibit many of the features of UC seem to be driven bacterially and have helped to unravel the pathogenic mechanisms underlying IBD. Overall, it appears that an imbalance of the mucosal immune system leads to the overproduction of inflammatory cytokines, release of reactive oxygen metabolites and infiltration of immune cells into the intestine, resulting in uncontrolled intestinal inflammation and tissue damage.

Myeloid-derived suppressor cells (MDSCs) constitute a diverse cell population made up of immature myeloid cells (IMCs), consisting of mostly progenitor cells of macrophages, granulocytes, dendritic cells and myeloid cells at early stages of differentiation. In mice, MDSCs are characterized by the co-expression of Gr-1 and CD11b markers, and are further divided into granulocytic and monocytic subset, defined as $CD11b^{+}Ly6G^{+}Ly6C^{low}$ and $CD11b^{+}Ly6G^{low}Ly6C^{+}$ cells, respectively. In humans, phenotypic characterization of MDSCs is challenging, due to lack of uniform criteria. Nevertheless, they are most commonly characterized by the expression of the myeloid marker CD33 and the lack of expression of the major histocompatibility complex (MHC) class-II molecule, human leukocyte antigen (HLA-DR) and several other lineage markers. Although most of our knowledge on the role of MDSCs in immune responses has been based on tumor bearing mice and cancer patients, increasing evidence has suggested their role in many pathological conditions, such as infections, transplantation and autoimmunity. Traditionally, MDSCs are considered as potent suppressors of immune responses through different mechanisms. However,

recent literature highlighted their plasticity, denoting proinflammatory potential of specific subsets emerging in specific microenvironment.

Despite the well-defined suppressive effects of MDSCs on T cell responses, their function in autoimmune diseases, like IBD, is controversial. In particular, the immunosuppressive function of MDSCs was suggested by several reports showing that $CD11b^{+}Gr1^{+}$ MDSCs are increased during intestinal inflammation. As is the case for murine colitis models, $CD14^{+}HLA\text{-}DR^{low}$ MDSCs with suppressive properties were also reported to be increased in the peripheral blood (PB) of IBD patients. On the other hand, recent studies supported a proinflammatory role of myeloid cells in experimental colitis, demonstrated that adoptively transferred colonic $Ly6C^{high}$ cells differentiated into inflammatory cells, contributing to intestinal inflammation.

In this PhD thesis, we investigated the immunomodulatory properties of MDSCs in experimental inflammatory colitis and T cell-mediated immune responses in IBD patients.

MDSCs (defined as $CD14^{+}HLA\text{-}DR^{-/low}CD33^{+}CD15^{+}$ cells) numbers were determined in PB from IBD patients. PB MDSCs function was assessed *in vitro*. Experimental colitis was induced upon 2,4,6-trinitrobenzene sulfonic acid (TNBS) treatment and MDSCs were characterized by flow cytometry. The *in vivo* suppressive potential of bone marrow (BM)-derived MDSCs (BM-MDSCs) was tested by using both depleting and adoptive transfer strategies.

MDSCs were enriched in the periphery of IBD patients during active disease. TNBS colitis induced amplification of MDSCs, particularly of the granulocytic ($Ly6G^{+}$) subset during the effector phase of disease. Of interest, BM-MDSCs potently suppressed $CD4^{+}$ T cell responses under steady state but failed to control colitis-associated immune responses *in vivo*. Mechanistically, under the colonic inflammatory milieu MDSCs switched phenotype (decreased proportion of $Gr1^{high}$ and increased numbers of $Gr1^{low}$) and downregulated CCAAT/enhancer-binding protein beta (CEBPβ) expression, a critical transcription factor for the suppressive function of MDSCs. In accordance with the murine data, human $CD33^{+}CD15^{+}$MDSCs from PB of IBD patients not only failed to suppress autologous T cell responses but instead enhanced T cell proliferation *in vitro*.

Our findings demonstrate an aberrant function of MDSCs in experimental inflammatory colitis and in IBD-associated immune responses *in vitro*. Delineation of

the mechanisms that underlie the loss of MDSCs function in IBD may provide novel therapeutic targets.

Keywords: MDSCs, BM-MDSCs, IBD, experimental colitis, CEBPβ

2. ΠΕΡΙΛΗΨΗ

Οι Ιδιοπαθείς Φλεγμονώδεις Νόσοι του Εντέρου (ΙΦΝΕ) είναι χρόνιες φλεγμονώδεις παθήσεις του πεπτικού σωλήνα, με υποτροπίαζοντα ή προοδευτικό χαρακτήρα που επηρρεάζουν εκατομμύρια ανθρώπων παγκοσμίως. Ο όρος ΙΦΝΕ περιλαμβάνει τη νόσο του Crohn και την ελκώδη κολίτιδα. Η ηλικία εμφάνισης της νόσου τοποθετείται συνήθως στη δεύτερη και τρίτη δεκαετία της ζωής, με την πλειονότητα των ασθενών να εμφανίζουν κλινική πορεία χαρακτηριζόμενη από υφέσεις και εξάρσεις. Αν και η ακριβής αιτιολογία των ΙΦΝΕ δεν έχει πλήρως αποσαφηνιστεί, θεωρείται νόσος πολυπαραγοντική. Φαίνεται ότι υπάρχει γενετική προδιάθεση, με υπεύθυνα συγκεκριμένα γονίδια που πιθανώς είναι ο πλέον σπουδαίος παράγοντας κινδύνου εμφάνισης των ΙΦΝΕ. Επιπλέον, η αλλαγή του μικροβιώματος δημιουργεί δυσβίωση μεταξύ ξενιστή και μικροβιώματος του εντέρου και αποτελεί σημαντικό παράγοντα του ξενιστή για την αιτιολογία της νόσου. Δεδομένα από πειραματικά μοντέλα κολίτιδας έχουν βοηθήσει σημαντικά στην κατανόηση των ανοσολογικών μηχανισμών που εμπλέκονται στην έναρξη και την εξέλιξη της νόσου. Συνολικά, φαίνεται ότι μία διαταραχή στο ανοσολογικό σύστημα του εντερικού βλεννογόνου οδηγεί σε υπερπαραγωγή φλεγμονωδών κυτταροκινών, απελευθέρωση μεσολαβητών οξειδωτικού στρες και διήθηση του εντέρου από φλεγμονώδη κύτταρα με αποτέλεσμα φλεγμονή του εντέρου και ιστική καταστροφή.

Τα κατασταλτικά κύτταρα μυελικής προέλευσης (Myeoid-derived suppressor cells-MDSCs) αποτελούν έναν ετερογενή πληθυσμό κυττάρων που περιλαμβάνει μακροφάγα, κοκκιοκύτταρα, δενδριτικά κύτταρα και κύτταρα μυελικής σειράς σε πρώιμα στάδια διαφοροποίησης. Στα ποντίκια, τα MDSCs χαρακτηρίζονται από την έκφραση των Gr-1 και CD11b δεικτών και περιλαμβάνουν δύο υποομάδες κυττάρων: αυτά με μορφολογία παρόμοια με εκείνη των κοκκιοκυττάρων και φαινότυπο $CD11b^{+}Ly6G^{+}Ly6C^{low}$ και αυτά με μορφολογία παρόμοια με εκείνα των μονοκυττάρων και φαινότυπο $CD11b^{+}Ly6G^{low}Ly6C^{+}$. Στους ανθρώπους, ο φαινοτυπικός προσδιορισμός των MDSCs συνιστά πρόκληση, λόγω της έλλειψης ενιαίων κριτηρίων. Συνηθέστερα, χαρακτηρίζονται απο την έκφραση του δείκτη CD33 της μυελικής σειράς και την έλλειψη έκφρασης τόσο του μορίου τάξης ΙΙ του μείζονος συμπλέγματος ιστοσυμβατότητας (MHC-II), HLA-DR, όσο και άλλων δεικτών που χαρακτηρίζουν τις υπόλοιπες κυτταρικές σειρές του μυελού των οστών. Αν και ο ρόλος των MDSCs έχει κυρίως μελετηθεί σε πειραματικά μοντέλα και

ασθενείς με καρκίνο, πρόσφατες μελέτες αναδεικνύουν το ρόλο αυτών σε πληθώρα παθολογικών καταστάσεων, όπως οι λοιμώξεις, η μεταμόσχευση και η αυτοανοσία. Παραδοσιακά, τα εν λόγω κύτταρα θεωρείται ότι καταστέλλουν την ανοσιακή απάντηση μέσω ποικίλων μηχανισμών. Ωστόσο, η πρόσφατη βιβλιογραφία επισημαίνει την πλαστικότητα αυτών των κυττάρων, δεδομένου ότι το εκάστοτε φλεγμονώδες περιβάλλον μπορεί να τους προσδώσει προφλεγμονώδεις ιδιότητες.

Αν και οι κατασταλτικές ιδιότητες των MDSCs στις διαμεσολαβούμενες απο Τ κύτταρα ανοσιακές απαντήσεις είναι καλώς καθορισμένες, ο ρόλος αυτών στα αυτοάνοσα νοσήματα, όπως οι ΙΦΝΕ, είναι αμφιλεγόμενος. Πιο συγκεκριμένα, η ανοσοκατασταλτική δράση των MDSCs υποστηρίζεται από πολλές εργασίες που αναδεικνύουν την αύξηση των $CD11b^{+}Gr1^{+}$ MDSCs σε πειραματικά μοντέλα κολίτιδας. Παρομοίως, αύξηση των κατασταλτικών $CD14^{+}HLA\text{-}DR^{low}$ MDSCs περιγράφεται και στο περιφερικό αίμα ασθενών με ΙΦΝΕ. Ωστόσο, πρόσφατες μελέτες επισημαίνουν τον προφλεγμονώδη ρόλο των κυττάρων της μυελικής σειράς σε πειραματικά μοντέλα κολίτιδας, καθώς αυτόλογη μεταφορά $Ly6C^{high}$ κυττάρων εντερικού βλεννογόνου οδηγεί σε διαφοροποίηση των τελευταίων σε κύτταρα που προάγουν τη φλεγμονή του εντέρου.

Στην παρούσα διδακτορική διατριβή, το ενδιαφέρον εστιάστηκε στον ανοσορυθμιστικό ρόλο των MDSCs σε πειραματικά μοντέλα κολίτιδας, καθώς και στις ανοσιακές απαντήσεις που διαμεσολαβούνται από τα Τ κύτταρα σε ασθενείς με ΙΦΝΕ.

Ο αριθμός των MDSCs (ταυτοποιούμενα ως $CD14^{+}HLA\text{-}DR^{-/low}CD33^{+}CD15^{+}$ κύτταρα) προσδιορίστηκε στο περιφερικό αίμα ασθενών με ΙΦΝΕ, ενώ η δράση αυτών αξιολογήθηκε με *in vitro* δοκιμασίες. Το πειραματικό μοντέλο που χρησιμοποιήθηκε ήταν αυτό της χημικά 2,4,6-trinitrobenzene sulfonic acid (TNBS) επαγόμενης κολίτιδας. Τα MDSCs χαρακτηρίστηκαν με τη μέθοδο της κυτταρομετρία ροής. Η *in vivo* ανοσοκατασταλτική δράση των MDSCs που καλλιεργήθηκαν από μυελό των οστών υγιών ποντικιών (BM-MDSCs) ελέγχθηκε τόσο με δοκιμασίες απαλοιφής όσο και αυτόλογης μεταφοράς.

Η μελέτη σε ανθρώπινα δείγματα ασθενών με ΙΦΝΕ, ανέδειξε αύξηση του αριθμού των MDSCs στο περιφερικό αίμα ασθενών με ενεργό νόσο. Παρομοίως, τα MDSCs, ιδιαίτερα ο υποπληθυσμός που χαρακτηρίζεται ως $Ly6G^{+}$, αυξήθηκε στα περιφερικά λεμφικά όργανα ποντικών με TNBS επαγόμενη κολίτιδα κατά την ενεργό φάση της

νόσου. Αντίθετα με τις αρχικές προσδοκίες, η αυτόλογη μεταφορά BM-MDSCs σε ποντίκια με TNBS κολίτιδα απέτυχε να ελέγξει τη φλεγμονή *in vivo*. Περαιτέρω μελέτη του μηχανισμού δράσης των MDSCs, έδειξε ότι η έκθεση των εν λόγω κυττάρων στο φλεγμονώδες περιβάλλον της κολίτιδας οδηγεί σε αλλαγή του φαινότυπου (μείωση των $Gr1^{high}$ και αύξηση των $Gr1^{low}$ κυττάρων) αυτών, καθώς και σε μειωμένη έκφραση της πρωτεΐνης CCAAT/enhancer-binding protein beta (CEBPβ), η οποία αποτελεί μεταγραφικό παράγοντα κλειδί στην ανοσοκατασταλτική δράση των MDSCs. Σε συμφωνία με τα αποτελέσματα της μελέτης στο πειραματικό μοντέλο κολίτιδας, MDSCs τα οποία απομονώθηκαν από περιφερικό αίμα ασθενών με ενεργό νόσο όχι μόνο δεν κατέστειλαν αλλά αντιθέτως ενίσχυσαν τον πολλαπλασιασμό αυτόλογων $CD4^+$ Τ κυττάρων *ex vivo*.

Συνολικά, τα δεδομένα αυτά αναδεικνύουν μια παράδοξη λειτουργία των MDSCs σε πειραματικά μοντέλα κολίτιδας, καθώς και στην *in vitro* ανοσιακή απάντηση ασθενών με ΙΦΝΕ. Η περαιτέρω κατανόηση των μηχανισμών που οδηγούν στην απώλεια του βασικού χαρακτηριστικού των MDSCs, που συνίσταται στην καταστολή των ανοσιακών απαντήσεων, θα μπορούσε να υποβοηθήσει στο σχεδιασμό νέων θεραπευτικών στόχων.

Λέξεις κλειδιά: MDSCs, BM-MDSCs, ΙΦΝΕ, πειραματική κολίτιδα, CEBPβ

3. ABBREVIATIONS

IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
UC	Ulcerative Colitis
MDSCs	Myeloid-derived suppressor cells
IMCs	Immature Myeloid Cells
DC	Dendritic cell
MHC	Major Histocompatibility Complex
HLA	Human Leukocyte Antigen
PB	Peripheral Blood
TNBS	2,4,6-trinitrobenzene sulfonic acid
BM	Bone marrow
BM-MDSCs	Bone marrow-derived myeloid-derived suppressor cells
CEBPβ	CCAAT/enhancer-binding protein beta
G-MDSCs	Granulocytic MDSCs
PMN-MDSCs	Polymorphonuclear MDSCs
M-MDSCs	Monocytic MDSCs
Lin	Lineage
eMDSCs	early stage MDSCs
ARG	Arginase
iNOS	Inducible Nitric Oxide Synthase
ROS	Reactive Oxygen Species
PNT	Peroxynitrate
ER	Endoplasmic Reticulum
TAMs	Tumor-associated macrophages
IRF	IFN Regulatory Factor
CFA	Complete Freund's Adjuvant
IFN	Interferon
IL	Interleukin
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
SCF	Stem Cell Factor
VEGF	Vascular Endothelial Growth Factor
Cox-2	Cyclooxygenenase

JAK	Janus Kinase
STAT	Signal transducer and activator of transcription
G-CSF	Granulocyte Colony-Stimulating Factor
TGFβ	Transforming Growth Factor β
TLR	Toll-like Receptor
NF-kB	Nuclear Factor kB
MyD88	Myeloid Differentiation Primary Response Gene(88)
CHOP	C/EBP homologous protein
TCR	T Cell Receptor
CCL	C-C motif Chemokine Ligand
Gal-9	Galectin-9
Tim-3	T cell immunoglobulin mucin-3
FoxP3	Forkhead box P3
RNS	Reactive Nitrogen Species
NO	Nitric Oxide
MS	Multiple Sclerosis
T1D	Type 1 Diabetes
SLE	Systemic Lupus Erythematosus
RA	Rheumatic Arthritis
AIH	Autoimmune Hepatitis
EAE	Experimental Autoimmune Encephalomyelitis
PD-L	Programmed Death-Ligand
PBMCs	Peripheral Blood Mononuclear Cells
ET	Extracellular trap
CIA	Collagen-induced Arthritis
PG	Proteoglycan
PGIA	Proteoglycan-induced arthritis
SF	Synovial Fluid
DSS	Dextran Sulfate Sodium
gp130	glycoprotein 130
TNFα	Tumor Necrosis Factor α
APC	Antigen Presenting Cell
LPS	Lipopolysaccharide
LP	Lamina Propria

TNP	Trinitrophenyl
NK	Natural Killer
NOD	Nonobese Diabetic (mice)
PEG2	Prostaglandin E2
IDO	Indoleamine 2,3-Dioxygenase
EP	E-Prostanoid
MOG	Myelin Oligodendrocyte Protein
MLN	Mesenteric Lymph Node
OVA	Ovalbumin
CREB	c-AMP response element-binding protein
JNK	c-Jun N-terminal Kinase
ERK	Extracellular signal-regulated kinase
CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor

4. INTRODUCTION

4.1. MDSCs

4.1.1. Definition of MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population consisting of myeloid precursor cells and immature myeloid cells (IMCs) with immunoregulatory properties. Under steady state, IMCs generated in the bone marrow differentiate into mature granulocytes, macrophages or dendritic cells (DCs). However, various cytokines and soluble factors released during pathological conditions, including cancer, infection, trauma, sepsis, transplantation and autoimmune diseases, trigger proliferation of IMCs and subsequently inhibit their normal differentiation. This results in expansion of MDSCs population, which then migrates to peripheral lymphoid organs and tissues, exerting their effect on other cell subsets (**Figure 1**) (1)

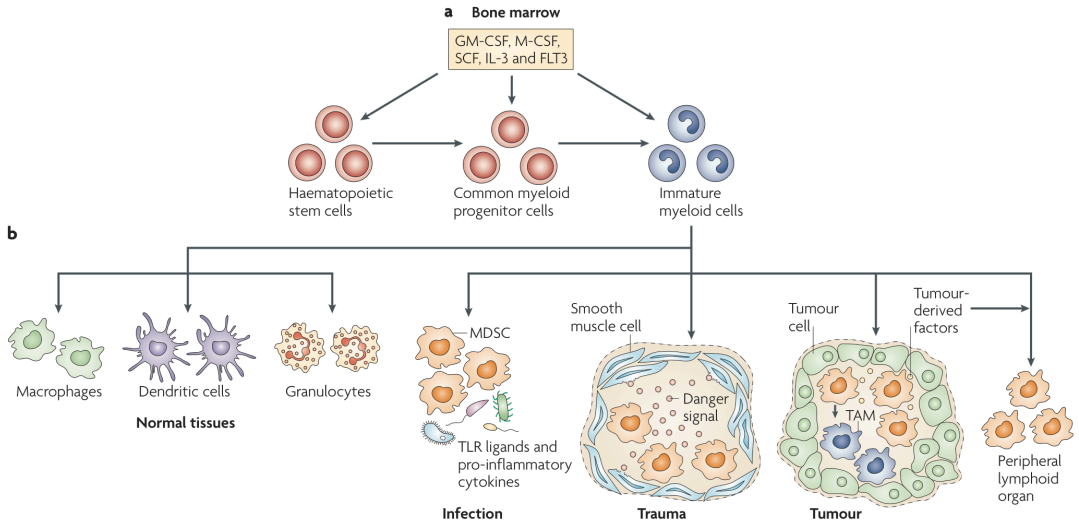


Figure 1. The origin of MDSCs. a. Immature myeloid cells (IMCs) are part of the normal process of myelopoiesis, which takes place in the bone marrow and is controlled by a complex network of soluble factors, including cytokines (such as granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), stem-cell factor (SCF), interleukin-3 (IL-3) and FMS-related tyrosine kinase 3 (FLT3)) and cell-expressed molecules (such as Notch; not shown). Haematopoietic stem cells differentiate into common myeloid progenitor cells and then into IMCs. **b.** Normally, IMCs migrate to different peripheral

organs, where they differentiate into macrophages, dendritic cells or granulocytes. However, factors that are produced during acute or chronic infections, trauma or sepsis, and in the tumour microenvironment promote the accumulation of IMCs at these sites, prevent their differentiation and induce their activation. These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells (MDSCs). MDSCs also accumulate in peripheral lymphoid organs in response to tumour-derived factors. TLR, Toll-like receptor. (source: Gabrilovich, D. I., and S. Nagaraj, *Nature Rev Immunol*, 2009)

4.1.2. MDSCs surface markers and subsets

In mice, MDSCs were initially defined as cells co-expressing the myeloid cell-lineage differentiation antigen Gr1 and CD11b (1). More recently, based on the expression of lymphocyte antigens Ly6C and Ly6G, MDSCs can be subdivided into two major fractions: $CD11b^{+}Ly6G^{+}Ly6C^{low}$ cells, with a granulocyte-like morphology, identified as granulocytic or polymorphonuclear-MDSCs (G-MDSCs or PMN-MDSCs), and $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells, with a monocytic-like morphology, known as monocytic-MDSCs (M-MDSCs) (**Figure 2**). Furthermore, other surface markers, including CD80 (B7.1) (2), CD115 (macrophage-colony stimulating factor receptor) (3) (4), CD124 (interleukin-4 receptor a chain) (4) and CD49d (5) have been used to characterize certain subsets of MDSCs. In naïve mice, $Gr1^{+}CD11b^{+}$ cells constitute 20-30% of bone marrow cells and 2-4% of splenocytes, while they are absent from lymph nodes (1). In contrast, the proportion of splenic MDSCs increases to 10-15% or more in tumor models whereas the increase of cells in bone marrow and peripheral blood of mice is usually less than twofold (6). Notably, in most tumor models, it is predominantly (70-80%) the granulocytic subset that expands.

In humans, phenotypic characterization of MDSCs is even more difficult, due to lack of uniform criteria. Initially, MDSCs were defined as $CD33^{+}HLA\text{-}DR^{-/low}$ cells that lack the expression of markers characteristic of mature myeloid and lymphoid cells (lineage negative (Lin^{-}) cells) (7). Similar to murine MDSCs, more recent reports suggest the subdivision of the whole population into $CD15^{+}CD14^{-}$ and $CD15^{-}CD14^{+}$ cells, representing the granulocytic (G-MDSCs) and monocytic subset (M-MDSCs), respectively. M-MDSCs express the myeloid marker CD33 while G-MDSCs display $CD33^{dim}$ staining. $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ cells defined as "early-stage MDSCs" (e-

MDSCs) contain mixed groups of MDSCs comprising more immature progenitors (6) (8) (**Table 1**). IMCs are barely detected in the peripheral blood of healthy subjects, constituting ~0.5% of peripheral blood mononuclear cells (1).

Table 1. Phenotype of MDSCs

Table 1 | Minimal phenotypic characteristics necessary to identify cells as MDSC.

Mouse	Phenotype	Human (in PBMC fraction)	Phenotype
Total MDSC (not sufficient for MDSC characterization)	Gr-1$^+$CD11b$^+$	Total (mixed) MDSC	Not clearly determined
PMN-MDSC	CD11b$^+$Ly6C^{lo}Ly6G$^+$	PMN-MDSC	CD14$^-$CD11b$^+$CD15$^+$ (or CD66b$^+$)
M-MDSC	CD11b$^+$Ly6C^{hi}Ly6G$^-$	M-MDSC	CD11b$^+$CD14$^+$HLA-DR$^{low/-}$ CD15$^-$
eMDSC	Not clearly determined	e-MDSC	Lin$^-$ (CD3/14/15/19/56)/ HLA-DR$^-$/CD33$^+$

eMDSC, early-stage MDSC; MDSC, myeloid-derived suppressor cell; M-MDSC, monocytic-MDSC; PBMC, peripheral blood mononuclear cell; PMN-MDSC; polymorphonuclear-MDSC.
Although phenotype is the first necessary step for defining MDSC, please note that, it cannot be used as the sole parameter for distinction between PMN-MDSC and neutrophils and M-MDSC and monocytes.
It is important, wherever possible, to use cells from control mice or healthy donors as controls.

(source: Bronte, V. *et al*, *Nat Commun*, 2016)

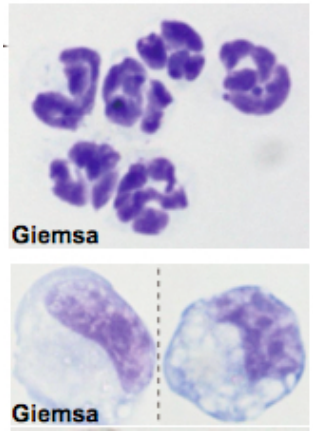


Figure 2. Granulocytic and Monocytic subsets of MDSCs. Giemsa staining of granulocytic (CD11b$^+$Ly6G$^+$Ly6C^{low}) (upper) and monocytic (CD11b$^+$Ly6G$^-$Ly6C^{high}) subset of MDSCs sorted from tumor infiltrates. (source: Sawanobori, Y. *et al*, *Blood*, 2008)

4.1.3. Relationship of MDSCs to other myeloid cells

MDSCs are pathologically activated myeloid cells. Although they are morphologically and phenotypically similar to neutrophils and monocytes, several lines of evidence support their unique nature.

1. Potent immunosuppressive character of MDSCs defines them as functionally different from neutrophils and monocytes. Mature neutrophils and monocytes cannot be converted to potent immunosuppressive cells *in vitro* by simply activating them with exogenous and endogenous danger signals or proinflammatory cytokines. In some cases, neutrophils actually promote antitumor response.

2. Murine MDSCs are characterized by specific proteome and transcriptome profiles. Human G-MDSCs have a different genomic profile from that of neutrophils in the same patient while neutrophils from healthy donors and cancer patients share similar gene expression.
3. Certain biochemical markers are associated with MDSCs as opposed to neutrophils and monocytes. These features include high levels of arginase (ARG) and inducible nitric oxide synthase (iNOS), increased reactive oxygen species (ROS) production, including superoxide, myeloperoxidase, hydroxyl peroxide and peroxynitrate (PNT). Furthermore, elevated endoplasmic reticulum (ER) stress response in MDSCs differentiates them from their control counterparts.
4. M-MDSCs can be distinguished from tumor-associated macrophages (TAMs) based on phenotypic characteristics. These include increased relative expression of F4/80, low to intermediate expression of Ly6C, low or undetectable expression of S100A9 protein, increased expression of interferon regulatory factor 8 (IRF8) and increased expression of CD115 (8).

4.1.4. Expansion of MDSCs in diverse pathologic conditions

Although most of our knowledge on the role of MDSCs in immune responses has been based on studies with tumor-bearing mice and cancer patients, increasing evidence has suggested their role in many pathological conditions, such as during infections, traumatic stress, sepsis and transplantation. Indeed, expansion of MDSCs was observed in mice primed with *Mycobacterium tuberculosis* in the form of complete Freund's adjuvant (CFA). Moreover, acute *Trypanosoma cruzi* infection, which induces T cell activation and increases the production of IFN-γ, also leads to the expansion of MDSCs (9) (10). A similar expansion of MDSCs has been reported during acute toxoplasmosis (11), polymicrobial sepsis (12), acute infection with *Listeria monocytogenes*, chronic infection with *Leishmania major* (13) and infections with helminthes (14) (15) (16) *Candida albicans* (17) or *Porphyromonas gingivalis* (18). MDSCs were also found to infiltrate the spleen and to suppress T cell function in a model of traumatic stress (19). Additionally, a significant transient increase in MDSCs numbers was also observed in normal mice following immunization with different antigens, including ovalbumin or peptide together with CFA, recombinant

vaccinia virus expressing IL-2, or staphylococcal enterotoxin A (20) (21). Finally, expansion of MDSCs is also associated with autoimmunity as discussed in paragraph 4.1.7.

4.1.5. Expansion and activation of MDSCs

Accumulation of MDSCs is controlled by a network of transcription factors and regulators separated in two partially overlapping groups: the first is responsible for expansion of MDSCs by promoting myelopoiesis and preventing differentiation whereas the second one mainly provides the necessary signals to acquire their suppressive phenotype.

Factors driving the expansion of MDSCs include granulocyte/macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor (SCF), IL-6, vascular endothelial growth factor (VEGF), coclooxygenase 2 (COX-2) and prostaglandins (22) (23) (24) (25) (26). These factors produced mainly by tumor microenvironment activate signal pathways that converge on Janus kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3) (27) (28). STAT3 signaling drives the expression of multiple anti-apoptotic proteins, which promote cell proliferation and inhibit their differentiation into mature cells (1) (29). Recent work has shed light on the pathways downstream of STAT3, identifying the transcription factor CCAAT-enhancer-binding protein beta (CEBPβ) as master regulator of differentiation of IMCs to functional MDSCs. Ablation of CEBPβ in the myeloid compartment reduced the expansion of MDSCs in the spleen of tumor-bearing mice. Moreover, CEBPβ deletion in bone marrow cells rendered these cells unable to differentiate *in vitro* into functional MDSCs (30). Interestingly, a direct link between STAT3 and CEBPβ has been reported. STAT3 controlled granulocyte colony stimulating factor (G-CSF)-responsive induction of CEBPβ expression in myeloid progenitor cells. Furthermore, G-CSF driven STAT3 activation stimulated c-myc expression by facilitating the binding of CEBPβ to c-myc promoter (31). Taken together, these data demonstrated that STAT3, at least partially, can induce expansion of MDSCs via up-regulation of CEBPβ.

Following expansion, MDSCs require a second signal generated either from activated T cells or bone marrow stromal cells, in response to viral and bacterial agents or products of cell death (12) to endow their suppressive function. These factors include IFN-γ, IL-1β, IL-4, IL-13, transforming growth factor-β (TGF-β) and Toll-like

receptors (TLRs) lignads and trigger signaling pathways orchestrated by STAT1, STAT6 and nuclear factor-kB (NF-kB) transcription factors. STAT1, the main transcription factor activated by IFN-γ and IL-1β mediated signaling, is implicated in the regulation of ARG1 and iNOS, molecules associated with MDSCs suppressive phenotype (4) (32) (33). Activation of STAT6 pathway in response to IL-4 and/or IL-13 binding to IL-4Ra is suggested to mediate suppressive function of MDSCs, by inducing both ARG1 and TGF-β production (34) (35) (36) (37) (38). Furthermore, TLR signaling via myeloid differentiation primary-response gene 88 (MyD88) and NF-kB upregulates intermediates with potential suppressive activity, during microbial and viral infections, as well as in trauma and sepsis (12) (39) (40). More recent data have linked the ER stress response pathway to MDSCs pathophysiology. MDSCs isolated from cancer patients and tumor-bearing mice overexpressed several markers of ER stress, including transcription factor CEBP-homologous protein (CHOP), the main mediator of ER stress response-induced apoptosis (41). Administration of an ER-stress inducer to tumor-bearing mice increased the accumulation of MDSCs and their immunosuppressive function (42). Interestingly, CHOP-deficient MDSCs not only lost their ability to suppress T cells but they even induced T cell proliferation, probably through CEBP*β* signaling impairment (43).

4.1.6. Mechanisms of MDSCs suppressive activity

MDSCs utilize a variety of mechanisms to exert their suppressive activity. These mechanisms could be divided into four groups (**Figure 3**).

The first type of mechanism is the depletion of amino acids essential for T cell growth and differentiation. More specifically, it includes L-arginine depletion through ARG1 activity (44) and L-cysteine deprivation via its consumption and sequestration (45). The depletion of these amino acids induces the loss of the ζ chain in the T cell receptor (TCR) and prevents upregulation of the cell-cycle regulator in T cells resulting in inhibition of T cell proliferation.

The second type of mechanism involves the generation of oxidation stress caused by the production of reactive nitrogen species (RNS) and ROS by MDSCs. RNS include nitric oxide (NO), a molecule generated by increased activity of iNOS, and able to inhibit IL-2 receptor signaling pathway by blocking the phosphorylation of JAK3 or STAT5 (46), or to directly induce T cell apoptosis (47). ROS including hydrogen

peroxide and PNT induce the loss of the CD3ζ chain (48) and blocks $CD8^+$ T cell activation by nitration and desensitization of TCR (49).

The third type of mechanism interferes with lymphocytes migration to lymph nodes or inflammation site. Expression of ADAM metallopeptidase domain 17 (ADAM17) by MDSCs induces the cleavage of L-selectin (CD62L) ectodomain expression on the surface of naïve $CD4^+$ and $CD8^+$ T cells resulting in inhibition of T lymphocytes homing to draining lymph nodes (50). PNT production by MDSCs results in nitration of C-C motif chemokine ligand 2 (CCL2), thereby preventing $CD8^+$ T cells chemotaxis to the tumor site (51). Furthermore, binding of galectin 9 (Gal-9) expressed on MDSCs with T cell immunoglobulin-3 (Tim-3) on lymphocytes induces T cell death (52). Finally, membrane contact dependent mechanisms and interaction with the natural killer (NK) receptor NKp30 are implicated in MDSC-mediated suppression of NK cells (53) (54) (55).

The fourth type of mechanism is the induction of $FoxP3^+$ Treg cells. The mechanisms underlying this process are not fully understood, but may involve CD40-CD40L interaction between MDSCs and T cells (56), soluble factors produced by MDSCs such as IFN-γ, IL-10 and TGF-β (3), and possibly ARG1 expression by MDSCs (38) . Interestingly, human $CD14^+HLA\text{-}DL^{-/low}$ MDSCs catalyze the transdifferentiation of $FoxP3^+$ Treg cells from Th17 cells via TGF-β and retinoic acid (57).

It is of note to mention that different subsets of MDSCs might use different mechanisms to suppress T cell proliferation. G-MDSCs express high levels of ROS and low levels of NO whereas M-MDSCs express low levels of ROS and high levels of NO. Both subsets express ARG1. Despite having different mechanisms of action, both subsets suppress T cell proliferation to an equal extent. The biological significance of such function difference between two MDSCs subsets remains to be addressed (1).

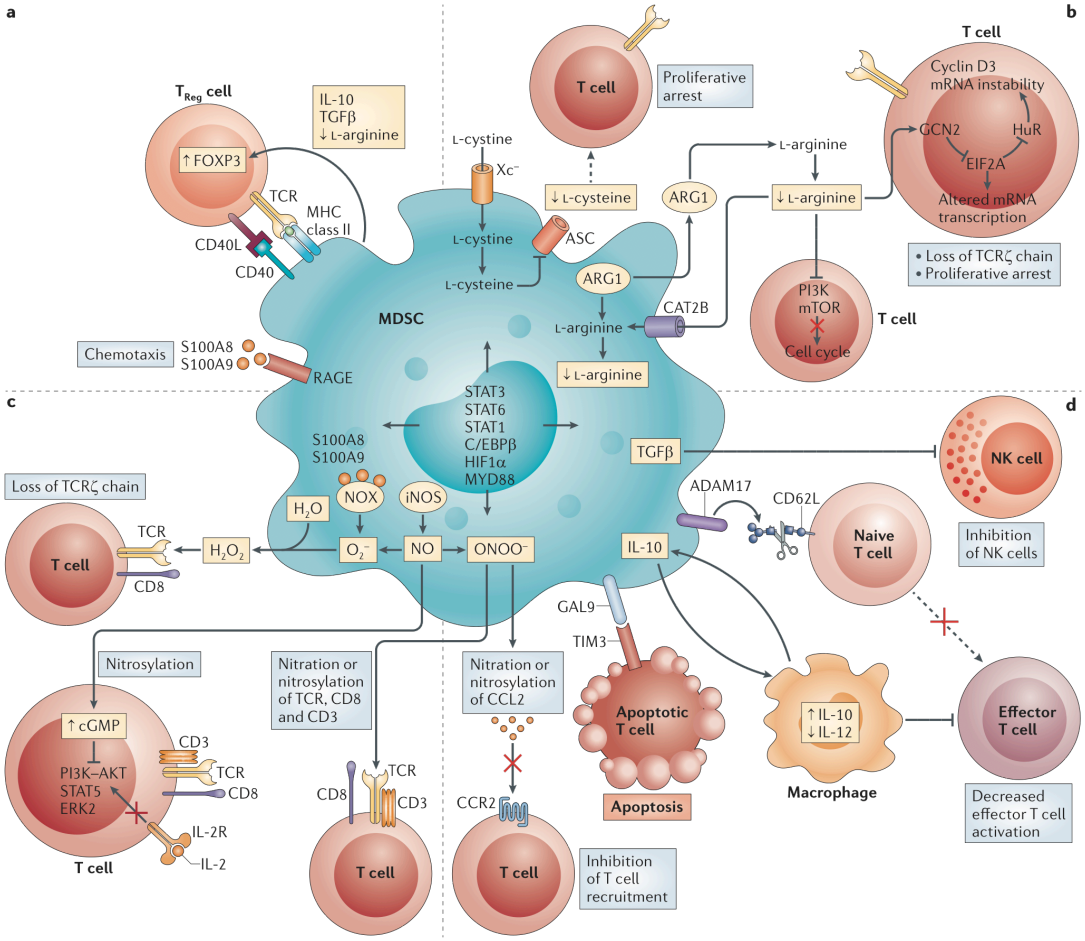


Figure 3. Mechanisms of MDSC-dependent inhibition of T cell activation and proliferation. a. Tumour-associated MDSCs induce the development of regulatory T (Treg) cells or expand existing Treg cell populations. The calcium-binding proteins S100A8 and S100A9 are involved in the chemotaxis of MDSCs and other myeloid cells; these effects are mediated in part through the activation of receptor for advanced glycation end-products (RAGE). At the same time, S100A8 and S100A9 along with gp91phox are part of the NADPH oxidase (NOX) complex that is responsible for the increased production of reactive oxygen species (ROS) by MDSCs. **b.** Tumour-associated myeloid cells deprive T cells of amino acids that are essential for their growth and differentiation. **c.** Tumour-associated myeloid cells release oxidizing molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Peroxynitrite causes nitration and nitrosylation of components of the T cell receptor (TCR) signalling complex, and H_2O_2 causes the loss of the TCR ζ-chain, thereby inhibiting T cell activation through the TCR. **d.** Tumour-associated myeloid cells can also interfere with T cell migration and viability. The metalloproteinase ADAM17 (disintegrin and metalloproteinase domain-containing protein 17) cleaves CD62L, which is necessary for T cell migration to draining lymph nodes, and galectin 9 (GAL9) can engage T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on T cells to induce apoptosis. ARG1, arginase 1; ASC, asc-type amino acid transporter; CAT2B, cationic amino

acid transporter 2 isoform 1 (l-arginine transporter); CCL2, CC-chemokine ligand 2; CCR2, CC-chemokine receptor 2; C/EBPβ, CCAAT/enhancer-binding protein-β; EIF2A, eukaryotic translation initiation factor 2A; ERK2, extracellular signal-regulated kinase 2; FOXP3, forkhead box P3; HIF1α, hypoxia-inducible factor 1α; HuR, Hu-antigen R (also known as ELAVL1); IL, interleukin; IL-2R, IL-2 receptor; iNOS, inducible nitric oxide synthase; mTOR, mammalian target of rapamycin; MYD88, myeloid differentiation primary-response protein 88; NK, natural killer; PI3K, phosphoinositide 3-kinase; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; Xc^{-}, cystine–glutamate transporter. (source: Gabrilovich, D.I. *et al*, *Nat Rev Immunol*, 2012)

4.1.7. MDSCs in autoimmunity

Most of our knowledge on the role of MDSCs in immune responses has been based on studies with tumor models and cancer patients. The role of MDSCs in autoimmune diseases is just starting to emerge.

We know that MDSCs are involved in several different autoimmune diseases, including multiple sclerosis (MS), type 1 diabetes (T1D), Systemic Lupus Erythematosus (SLE), rheumatoid arthritis (RA), autoimmune hepatitis (AIH) and inflammatory bowel disease (IBD).

Development of experimental autoimmune encephalomyelitis (EAE), the mouse model of MS has been correlated with accumulation of $CD11b^{+}Ly6C^{high}Ly6G^{-}$ cells in spleens, which induced apoptosis of activated T cells through NO production (58). A few years later, the same group showed the functional plasticity of MDSCs during the course of disease: MDSCs isolated before EAE onset were proinflammatory, while those isolated at EAE peak were suppressive (59). More recent study suggested a protective effect of $CD11b^{+}Ly6G^{high}Ly6C^{-}$ cells in this model. *In vivo* adoptive transfer of G-MDSCs, purified from an autoimmune environment generated by the injection of a self-Ag plus adjuvant, reduced the expansion of autoreactive T cells and inhibited pathogenic Th1 and Th17 immune responses in a programmed-death ligand-1 (PD-L1)/IFN-γ-dependent manner. The accumulation of G-MDSCs not only in the peripheral lymphoid organs but also at spinal cord highlighted that cells act at the target tissue as well (60). On the other hand, MDSCs isolated from mice with EAE facilitated Th17 cell differentiation in an IL-1β-dependent manner. Although MDSCs efficiently suppressed T cells *in vitro*, under Th17 polarizing conditions, they promoted generation of Th17 cells (61).

In different mouse models of diabetes, adoptive transfer of MDSCs prevented/delayed the development of disease (30) (62) (63). The protective effect of MDSCs are suggested to be mediated via multiple mechanisms, including NO- and IL-10-dependent mechanisms (64), as well as induction of anergy in autoreactive T cells and development of $FoxP3^+$ Treg cells (63). In humans, a recent study described increased numbers of MDSCs in peripheral blood mononuclear cells (PBMCs) of patients with T1D capable of T cell suppression in a contact-dependent manner (65).

In lupus-prone MRL-Fas^{lpr} mice, the frequency of MDSCs increased in both kidney and peripheral blood during disease progression. Their inhibitory effect on T cell proliferation *in vitro* mediated by ARG1 (66). Increased numbers of MDSCs, especially granulocytic subset, were also demonstrated in spleens of male lupus-prone (NZB x NZW) F1 mice compared to their female counterparts. These cells directly inhibited differentiation of naïve B cells into antibody-secreting cells *in vitro*. Notably, anti-Gr1 depletion increased the production of antinuclear autoantibodies only in male lupus-prone mice. Such findings pose the scenario of a male-driven inhibitory mechanism in B cell pathogenesis, delaying or preventing the development of the disease in otherwise genetically predisposed male (NZB x NZW) F1 mice (67). More recent study using the similar lupus model, demonstrated impaired expansion and immunosuppressive capacity of G-MDSCs due to induction of extracellular trap (ET) formation in the lupus inflammatory milieu (68). In humans, the frequency of MDSCs in the peripheral blood of active SLE patients was not only increased but also positively correlated with serum ARG1 levels, Th17 responses and disease severity as well. In the same study, the disease-promoting role of MDSCs was further assessed in a humanized SLE mouse mode (69).

In the collagen-induced arthritis (CIA) model, which resembles RA, accumulation of MDSCs in the spleen correlated with the disease course. MDSCs were found to inhibit proliferation of $CD4^+$ T cells and their differentiation into Th17 cells as well, through the activity of both NO and ARG1 (70). Furthermore, in proteoglycan (PG)-induced arthritis (PGIA), another model of RA, synovial fluid (SF) G-MDSCs potently suppressed DC maturation and T cell proliferation via NO- and ROS-mediated mechanisms. However, spleen MDSCs failed to exert suppressive activity, indicating the role of microenvironment in determining the functionality of this cell population (71). Contrary to the previous studies, a recent study demonstrated a pathogenic role of MDSCs in autoimmune arthritis. MDSCs expanded in arthritic

mice and correlated positively with disease severity by promoting Th17 immune response. Although splenic MDSCs displayed T cell suppressive capacity through cell-contact mechanism *ex vivo*, they promoted Th17 cell differentiation. Furthermore, elimination of MDSCs by using anti-Gr1 mAb decreased disease severity along with Th17 immune response whereas adoptive transfer of M-MDSCs aggravated disease phenotype. In accordance with murine data, the frequency of MDSCs was not only increased in the peripheral blood but also in SF of active RA patients. Of note, the latter was positively correlated with IL-17A levels (72). Increased numbers of MDSCs in PBMCs of RA patients was also described in another study. However the correlation between MDSCs and Th17 cells was negative indicating a reciprocal inhibition (73).

Accumulation of MDSCs has also been demonstrated in TGF-$\beta1^{-/-}$ mice, which spontaneously develop AIH. Isolated $CD11b^{+}Ly6C^{high}Ly6G^{-}$ MDSCs from livers efficiently suppressed $CD4^{+}$ T cells *ex vivo* via a mechanism dependent on cell-cell contact, NO and IFN-γ. However, this study did not examine their *in vivo* effect (74). Subsequent studies using different models of experimental hepatitis, identified infiltration of liver with different subsets of MDSCs that expressed different suppressive molecules. Canabidiol or IL-25 increased induction of hepatic MDSCs, in favor of monocytic subset that performed its immunosuppressive activity *in vitro* in an arginase-dependent manner (75) (76). M-MDSCs expansion but also *in vivo* suppressive activity via an iNOS and/or ARG1-driven mechanism was described in two other studies (77) (78).

MDSCs were also described in a $CD8^{+}$ T cell-mediated model of IBD. The frequency of NOS2- and arginase-expressing $CD11b^{+}Gr1^{+}$ MDSCs with immunosuppressive function was increased in the spleen and intestine of colitic mice. Furthermore, co-transfer of transgenic T cells with MDSCs ameliorated intestinal inflammation. Most importantly, this study is the first one that investigated the role of MDSCs in patients with IBD. The frequency of M-MDSCs was increased in the peripheral blood of IBD patients, whereas coculture of them with autologous PBMCs suppressed proliferation and cytokine production by the later population (79). Murine data were also confirmed in dextran sodium sulfate (DSS)- and TNBS-induced colitis models. Increase of MDSCs in the spleen and/or intestine correlated with the severity of intestinal inflammation, while adoptive transfer of either splenic or *in vitro* generated MDSCs ameliorated enterocolitis (80) (81) (82). Furthermore, a recent study using

gp130$^{757F/F}$ mice, characterized by systemic hyperactivation of STAT3 and associated with protection from experimental colitis determined mechanisms for such protection and compared them to mice with myeloid specific STAT3 deficiency. Interestingly, the resistance to colitis in gp130$^{757F/F}$ mice was associated with myeloid cell specific STAT3 activation, MDSCs expansion in colon, increased ARG1 expression and production of the protective cytokines IL-19 and IL-33 in the colon (83). In contrast with the above studies, compelling evidence support a proinflammatory role of MDSCs in experimental IBD. Adoptively transferred Ly6C^{high} cells aggravated DSS colitis through differentiation into TNF-α secreting proinflammatory dendritic cells (84). Similarly, Ly6C^{high} cells recruited into the colon and converted into inflammatory DCs and macrophages, when transferred to RAG$^{-/-}$ mice, a murine model of chronic gut inflammation (85). Additionally, CD11b^{+}Ly6G^{+} cells isolated from chronically inflamed colons, functioned as antigen-presenting cells (APCs) and triggered T cell proliferation (86). In humans data are rather limited. Except from the study conducted by Haile *et al*, one more recent study confirmed the increased levels of M-MDSCs in the peripheral blood of IBD patients compared to control cohort (87). Collectively, these data calls into question the actual role of MDSCs in the pathogenesis of autoimmune diseases and support that local microenvironment may dictate the fate of MDSCs, either to promote or suppress T cell functions.

Table 2. Pros and Cons of MDSCs in IBD

Function of MDSCs	Model	Role	Reference
Immunosuppression	2,4,6-TNBS-induced colitis	Adoptive transfer of $CD11b^+Gr\text{-}1^+$ MDSCs decreased intestinal inflammation, levels of IFN-γ, IL-17 and TNF-α	Guan Q, 2013
	DSS-induced murine colitis	Anti-Gr1 antibody treatment exacerbated the DSS-induced colitis	Zhang R, 2011
	$CD4^+CD45RB^{high}$ T cell transfer-induced chronic colitis in RAG-$1^{-/-}$ mice	$Ly6C^{high}$ monocyte-derived cells restrained Th1 cell responses and promote generation of $FoxP3^+$ Tregs and Th17 cells	Kurmaeva E, 2014
	HA-specific $CD8^+$ T cell transfer-developed colitis in VILLIN-HA mice	Transfer of $CD11b^+Gr\text{-}1^+$ MDSCs ameliorated intestinal inflammation	Haile LA, 2008
	Spontaneous developed chronic colitis in $IL10^{-/-}$ mice	Resveratrol-induced $CD11b^+Gr\text{-}1^+$ cells attenuated T cell proliferation, and reduced IFN-γ and GM-CSF production by lamina propria derived T cells in vitro	Singh UP, 2012
Pro-inflammation	DSS-induced murine colitis	$CD11b^+CD14^+CX3CR1^+$ lamina propria DCs were derived from transferred $Ly6C^{high}$ monocytes contributed to severe intestinal inflammation in TNF-α dependent manner	Varol C, 2009C
	$CD4^+CD45RB^{high}$ T cell transfer-induced chronic colitis in $RAG^{-/-}$ mice	Colonic Mac-$1^+Ly6C^{int}Gr\text{-}1^+$ cells induced T cell activation/proliferation and pro-inflammatory cytokines, including IFN-γ, IL-17, TNF-α, and IL-1β production in vitro	Ostanin DV, 2012
	$CD4^+CD45RB^{high}$ T cell transfer-induced chronic colitis in $RAG^{-/-}$ mice	$Ly6C^{high}$ monocytes differentiated into $CD103^-$ $CX3CR1^{int}CD11b^+$ DCs, and produced high levels of pro-inflammatory cytokines, including IL-12, IL-23, iNOS, and TNF, in the colon	Rivollier A, 2012

TNBS, trinitrobenzene sulfonic acid; IFN-γ, interferon γ; IL-17, interleukin 17; TNF-α, tumor necrosis factor α; DSS, dextran sulfate sodium; GM-CSF, granulocyte-macrophage colony-stimulating factor; DCs, dendritic cells; RAG-1, recombination activation gene-1; iNOS, inducible nitric oxide synthase. (source: Kim, Y.J. *et al*, *Intest Res*, 2015)

4.1.8. MDSCs: friend or foe in autoimmunity?

Given the diversity of function of MDSCs in autoimmune models it is rather difficult to develop a unifying hypothesis explaining their role in autoimmune diseases. Generally speaking, $CD11b^+Gr1^+$ cells isolated from autoimmune inflammatory environments appear to potently inhibit T cell activation and proliferation when tested *in vitro*, through various mechanisms. Somewhat surprisingly, this seems not to be the case *in vivo*. Where tested, spontaneously arising endogenous MDSCs appear to serve as pathologic effectors of the disease, since they are ineffective at ameliorating or even exacerbate the immune response. On the other hand, exogenously applied MDSCs control autoimmune pathology.

To incorporate these controversial findings, recent studies suggest a functional polarization of MDSCs dictated by inflammatory milieu. By this model, endogenous MDSCs that recruited to an autoimmune/inflammatory environment possess

proinflammatory and immunostimulatory activities, while exogenously delivered MDSCs are characterized by their suppressive and anti-inflammatory function. Perhaps inflammatory factors prevent MDSCs from exerting their inhibitory role, while the removal of MDSCs from this environment allows the suppressor phenotype to emerge, rendering them functional upon readministration.

Consequently, it is proposed that the dysfunction of MDSCs *in vivo* may be a driving factor for autoimmune inflammatory response. These cells accumulate in response to inflammation, but fail to control unwanted immune responses, resulting in further inflammation, and additional recruitment of dysfunctional MDSCs (88).

4.2. Inflammatory Bowel Diseases (IBD)

4.2.1. IBD pathogenesis

IBD is defined as a chronic, relapsing intestinal inflammation that results from an aberrant immune response to intraluminal bacterial antigens in genetically susceptible individuals. This inflammatory condition includes two major forms known as Crohn's disease (CD) and ulcerative colitis (UC) (89) (90). To date, the precise cause of IBD remains poorly understood, and multiple factors are considered to be involved in the pathogenesis, including interactions among genetic factors, commensal microbiota, and host immune system (91).

In IBD, pathogenic bacteria or commensal microbes disrupt host epithelial barrier function, triggering the recruitment and activation of innate immune responses and colitogenic $CD4^+$ T cells. Adaptive responses are affected by a combination of resident and recruited cell populations. These include a mixture of T cells dominated by Th1, Th17 or Th2 cells, and the presence of regulatory cells, of either lymphoid or myeloid origin.

Traditionally, Th1 cells, together with their proinflammatory cytokines (e.g. IFN-γ) and TNF-α, was thought to drive the pathogenesis of CD, while UC was considered to exhibit a Th2 profile, characterized by enhanced production of IL-5 and IL-13 (92).

However, recent studies underline the role of Th17 cells (93) (94) (95), a subpopulation that expand in response to IL-23, and contract by transcription factors required for both Th1 and Th2 cells (96). The role of IL-17 remains controversial, with some studies suggesting a pathogenic role, while others showing a protective role (97). However, several studies have shown that IL-17A triggers and amplifies

inflammatory pathways during IBD course, and its inhibition might be of benefit for a subset of IBD patients (98).

Several lines of evidence highlight the essential role of Treg cells in maintaining intestinal homeostasis and suppressing intestinal inflammation. In the absence of proinflammatory mediators, TGF-β promotes the differentiation of induced Treg (iTreg) cells associated with immunosuppression and regulation. On the other hand, an inflammatory environment, rich in IL-6, a cytokine produced by the activated innate immune system, and TGF-β inhibits the generation of iTreg cells, and facilitates the differentiation of Th17 cells (99) (**Figure 4**).

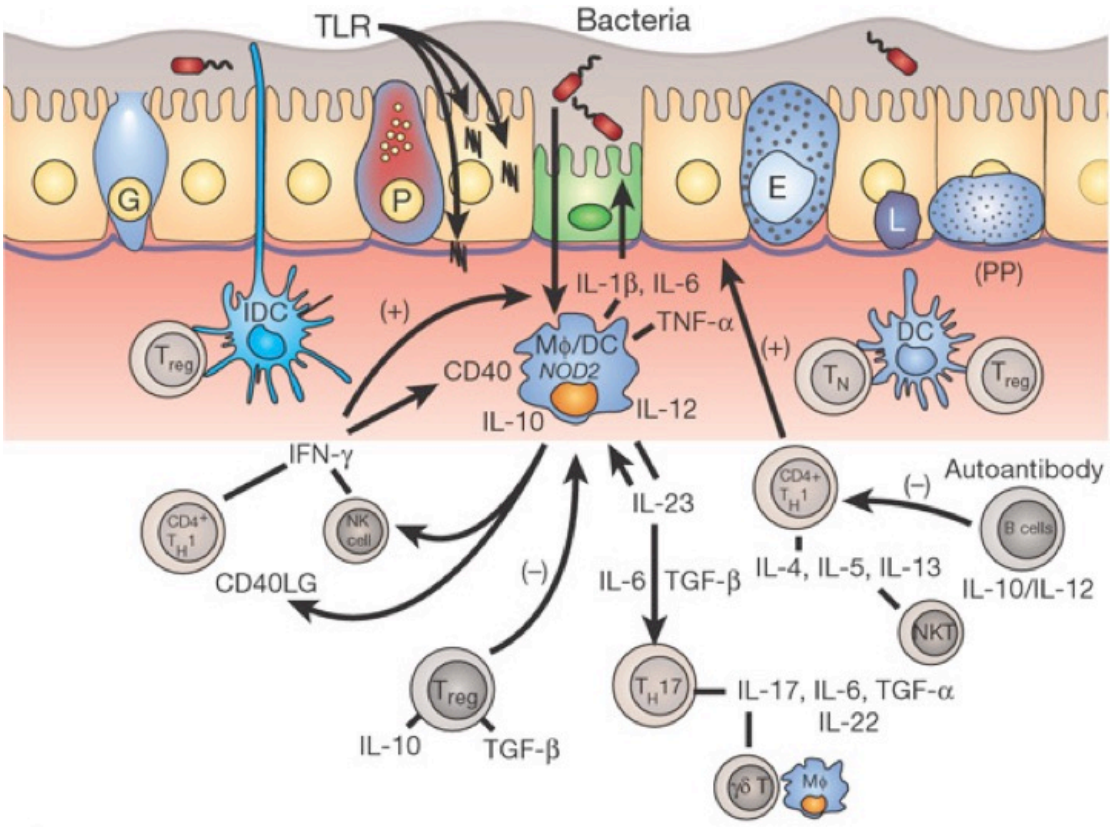


Figure 4. **Mucosal immune responses to luminal flora in IBD.** Mucosal immune response initiated by microbial sensing systems activates adaptive immune responses. Pathogenic bacteria or commensal microbes in genetically susceptible hosts disrupt epithelial barrier function, triggering the recruitment and activation of innate immune responses and colitogenic $CD4^+$ T cells. Depicted cells and cytokines imply that multiple components are involved in controlling mucosal immune responses in physiological and pathological states of inflammation. DC, dendritic cell; IgA, immunoglobulin A; E, enterocyte; G, goblet cell; L, lymphocyte; IDC, immature dendritic cell; T_N, T cell (naïve); MLN, mesenteric lymph node;

Mw, macrophage; P, Paneth cells; PP, Peyer's patches; NK, natural killer. (source: Xavier, R.J. *et al*, *Nature*, 2007)

Besides these alterations in the lymphoid compartment, the homeostasis of other cell populations is disrupted during the inflammatory process underlying IBD as well. Among those are MDSCs, which is the focus of the present study. Since their identification in mouse models of colitis and IBD patients have already been described, it would be interesting to discuss here the interplay between MDSCs and Th1/Th17 cells. Numerous studies support the involvement of IFN-γ in the induction of suppressive activity of MDSCs. Administration of IFN-γ in conjuction with LPS expands MDSCs population both *in vitro* and *in vivo* (40). IFN-γ produced by activated T cells upregulates the expression of genes, such as Arg-1 and iNOS, implicated in the inhibitory activity of MDSCs (4). In line with this observation, several studies indicate that neutralization of IFN-γ reverses inhibition of T cell proliferation when cocultured with MDSCs (4) (46) (58) (74). Similarly, coculture of MDSCs with IFN-$\gamma^{-/-}$ T cells abrogates suppressive potential of MDSCs (4). In chronic colitis model, neutralization of IFN-γ restores T cell proliferation when they are cocultured with splenic isolated MDSCs. However, suppression of T cell proliferation by lamina propria (LP) MDSCs is independent of IFN-γ (100).

Furthermore, there are studies suggesting that Th17 cells and associated cytokines may be directly involved in expansion and accumulation of MDSCs. IL-17 plays an important role in the migration of both neutrophils and monocytes from the BM to the blood (101) (102), while it is also a potent stimulator of myelopoiesis along with GM-CSF (103). Furthermore, IL-6, being involved in differentiation of Th17 cells, drives emergency granulopoiesis (104) (**Figure 5**).

Collectively, these data demonstrate the complex interactions between immune cells that affect the onset, perpetuation and relapses in human IBD.

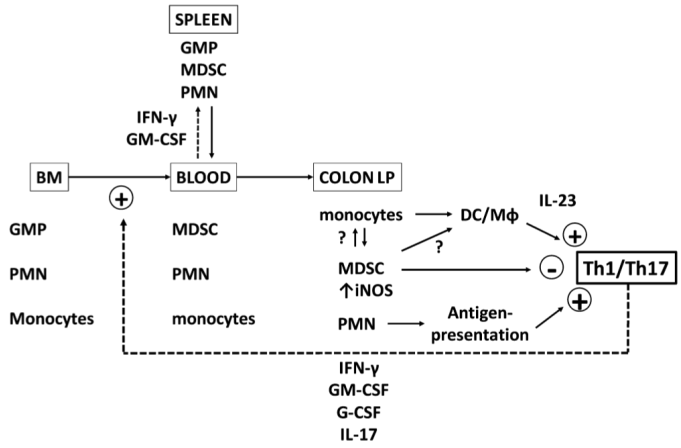


Figure 5. **Th1, Th17 and MDSCs network in IBD.** In chronic colitis, inflammatory cytokine milieu promotes myelopoiesis by stimulating production of myeloid precursors in the BM and by stimulating extramedullary hematopoiesis in the spleen. Circulating myeloid cells are recruited to colon LP or can be sequestered by spleen, which acts as temporary myeloid cell storage. Recruited myeoid cells undergo a series of phenotypic and functional changes: polymorphonuclear neutrophils (PMNs) acquire antigen-presenting functions and stimulate cytokine production by effector Th1/Th17 cells. Inflammatory monocytes are recruited into colon LP and differentiate into proinflammatory macrophages or dendritic cells (DC). MDSCs phenotypically resemble inflammatory monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{high}$), although these cells express iNOS and arginase-1, suggesting a more activated state. MDSCs are unable to suppress activated/effector Th1/Th17 cells and instead may also differentiate into proinflammatory macrophages/DC. Production of IL-12 and IL-23 by these cells promotes generation and/or stabilization of Th1 and Th17 cells, respectively. GMP: granulocyte-monocyte progenitor. (source: Ostanin, D.V. *et al*, *Inflamm Bowel Dis*, 2013)

4.2.2. TNBS-induced colitis-experimental model of IBD

Several murine models of experimental colitis have been developed in order to investigate the immunopathogenesis of IBD. TNBS-induced colitis is one of the most widely used and best described chemically-induced mouse models of intestinal inflammation for various reasons. The onset and duration of inflammation is immediate and controllable, whereas there are no artificial genetic deletion or manipulations that are usually not found in human IBD.

TNBS is a chemical compound that haptenates colonic autologous or microbiota proteins with trinitrophenyl (TNP) groups and renders them immunogenic to the host immune system.

Colitis is induced by intrarectal injection of TNBS diluted in ethanol in susceptible mouse strains. Ethanol is a prerequisite so that the intestinal epithelial barrier is disrupted and lamina propria cells are exposed to bacterial components (105).

Once administered via the rectum, TNBS induces T cell mediated immune responses characterized by massive transmural infiltrations of T cells and macrophages and thickening of the colon wall. This histopathologic picture is accompanied by clinical manifestations, such as bloody diarrhea, weight loss and rectal prolapse that resemble human IBD. The chronic stage of the disease is associated with an activation of the mucosal immune system and an increase in the number of infiltrating lymphocytes, especially $CD4^+$ T cells in the lamina propria. Lamina propria T cells isolated from colitic mice produce high levels of IFN-γ and IL-2, consistent with a Th1 immune response. Since treatment against TNF-α and anti-IL-12 ameliorates the disease, it has been suggested that a positive feedback loop between macrophages producing T cell activating IL-12 upon contact with bacterial antigens and T cells producing macrophage activating IFN-γ contributes to the chronicity of inflammation (106). On the other hand, TGF-β and IL-10 seem to play an important role for the downregulation of the inflammatory process (107). These immune responses mediated TNBS-induced intestinal inflammation, along with the histopathological aspect are consistent with those observed in patients with CD.

It is worthy to note here that induction of TNBS model depends on the genetic background of the animal strain. In particular, SJL and BALB/c are susceptible, whereas C57BL/6 and C57BL/10 mice are highly resistant to TNBS colitis (108). The susceptibility seems to be related to a genetically determined high IL-12 response to the LPS locus on chromosome 11 (109).

4.3. MDSCs as a new form of cell-based therapy

Considering the potent immune suppression that can be exerted by MDSCs, these cells promise to be useful in treating conditions of overwhelming immune activation. Autoimmune diseases are mainly characterized by an apparent activation of the immune system against self-antigens, leading in many cases to tissue damage and organ failure. Consequently, MDSCs could be harnessed as a cellular therapy for such diseases, at least theoretically. However, several questions remain to be addressed before their use as a treatment modality in autoimmunity.

1. Determine the conditions for optimizing the *in vitro* expanded cells, which must be stabilized and retain their suppressive activity so that be suitable for immunotherapy-based adoptive transfer. Recently, several groups have shown the *in vitro* generation of MDSCs originating from various cell types (embryonic and hematopoietic stem cells, BM cells, and PBMCs) by using different induction methods. Following, only those have been tested in autoimmune disease models will be described.

Rössner *et al*. developed a method for the *in vitro* generation of MDSCs by culturing BM cells with GM-CSF. *In vitro* generated MDSCs are $CD11b^{+}Gr1^{low}CD11c^{-}$ with ring-shaped nuclei. MDSCs generation is dependent on concentration of GM-CSF and duration of culture. In particular, high concentrations of GM-CSF favors MDSCs development after 3-4 days when they coincide with neutrophils in the culture while under low GM-CSF concentrations their generation requires 8-10 days when the cells appear with immature DCs (**Figure 6**). These cells share many features with their *ex vivo* isolated counterparts. They are potent inhibitors of $CD4^{+}$ and $CD8^{+}$ T cells proliferation via cell-cell contact, NO production and IFN-γ dependent mechanisms (110). The application of such cells suppressed the development of autoimmune diabetes in non-obese diabetic (NOD)-mice (62).

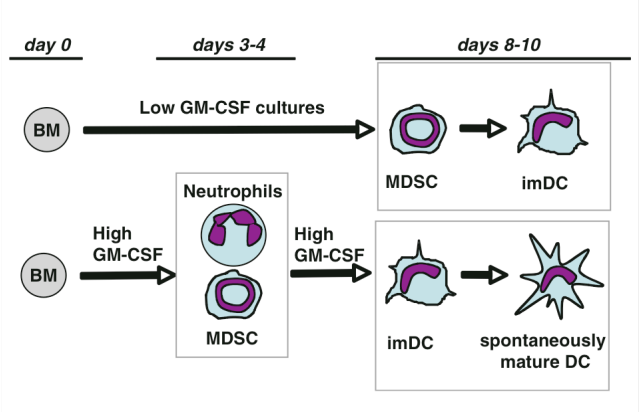


Figure 6. MDSC appear transiently in bone marrow-derived DC cultures, but at different time points, dependent on the protocol. Murine bone marrow cells cultures with GM-CSF give rise to MDSC under low dose GM-CSF conditions at days 8–10, coinciding with immature DC generation (Lutz MB, 2000). In contrast, high dose GM-CSF conditions favor a more rapid MDSC development at days 3–4 (Rössner S, 2005) at the same time of neutrophil appearance in the culture. If MDSC are not activated at day 3–4 they further develop into dendritic cells (Lutz MB K. N., 1999). (source: Ribechini, E. *et al*, *Med Microbiol Immunol*, 2010)

Similarly, Kurko *et al*. demonstrated that BM cells cultured in the presence of GM-CSF, IL-6, and G-CSF gave rise to an MDSC-like cell population with greater phenotypic heterogeneity than those isolated from synovial fluid of arthritic mice. BM-MDSCs profoundly suppressed both antigen-specific and non-specific T cell proliferation primarily in an NO-dependent manner. The injection of BM-MDSCs into mice with PGIA ameliorated arthritis and reduced PG-specific T cell responses and serum antibody levels (111).

Furthermore, Obermajer *et al*. used human PBMCs as a potential cellular source for MDSCs generation. Addition of prostaglandin E2 (PGE2), which induces endogenous COX-2 expression in cultured moncytes blocked their differentiation into $CD1a^+$ DCs and upregulated the expression of indoleamine 2,3-dioxygenase 1 (IDO1), IL-4Ra, iNOS2, and IL10, well known suppressive mediators of MDSCs. The intact COX-2-PGE2 axis is essential for the functional stability of the *de novo* generated MDSCs. Interestingly, selective E-prostanoid receptor (EP) 2- and EP4-agonists, but not EP3/1, also promoted MDSCs development, suggesting that other activators of the EP2/4 signaling pathway may be used to induce the generation of suppressive cells (**Figure 7**) (112).

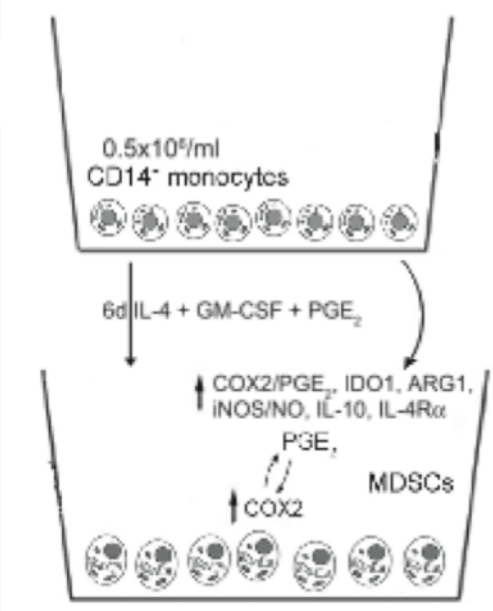


Figure 7. Prostaglandin E_2-induced positive cyclooxygenase 2-prostaglandin E_2-E-prostanoid receptor 2/4 feedback loop allows for ex-vivo generation of high numbers of myeloid-derived suppressor cells and their functional stability. Prostaglandin E_2 (PGE) (via E-prostanoid receptor (EP)2- and EP4-dependent signals) drives the early induction of

cyclooxygenase (COX)2 in local myeloid cells (monocytes, macrophages, immature dendritic cells (iDCs)), promoting their production of suppressive factors (indoleamine 2,3-dioxygenase (IDO) 1, IL-10, arginase 1, nitric oxide synthase (NOS)2, and PGE_2 itself, and acquisition of suppressive functions. These processes are further amplified by the de novo production of endogenous PGE, now produced at high levels by (MDSCs) themselves, thereby creating a positive feedback loop leading to persistence of MDSCs. The key role of the EP2- and EP4-mediated COX2-PGE_2 feedback to control multiple aspects of MDSCs function provides convenient targets to generate MDSC-associated immune regulation in tolerogenic therapies. (source: Obermajer, N. and P. Kalinski, *Transplant Res*, 2012)

2. Identification of specific markers utilized for their isolation. Overall, current data suggest that MDSCs are not a defined subset of cells, but rather a group of phenotypically heterogeneous myeloid cells with common activity. The known MDSCs markers are not MDSCs specific, but they are shared with other myeloid lineage cells, such as monocytes and neutrophils. Indeed, the lack of unifying criteria creates ambiguity in the definition and discrepancies in functional activity of these cells observed by different groups. This issue could be resolved by the identification of more specific markers that allow for discriminating between MDSCs and other cells of myeloid origin and defining more narrowly suppressive subsets of these cells. Recently, a novel molecule of MDSCs, CD49d, was suggested to be such a marker. In particular, $CD49d^+$ subset of MDSCs was mainly monocytic and strongly suppressed antigen-specific T cell proliferation in an NO-dependent manner, similarly to $Gr1^{-/int}$ MDSCs. $CD11b^+CD49d^+Gr1^+$ MDSCs were more potent suppressors of antigen-specific T cell responses than $CD11b^+CD49d^-Gr1^+$ MDSCs (5). It is likely that in the next few years more markers of suppressive subsets of MDSCs will be determined, which would help to clarify the biology of MDSCs in pathologic conditions.

3. Pinpoint the exact MDSCs population that confers immunosuppressive activity. In an EAE model, G-MDSCs from myelin oligodendrocyte glycoprotein (MOG)-immunized mice were found to express high levels of PD-L1, a costimulatory molecule that negatively regulates T cell proliferation. Interestingly, *in vivo* transfer of G-MDSCs inhibited autoantigen-priming of Th1 and Th17 cells, leading to delayed disease onset and amelioration of EAE phenotype, as well (60). On the other hand, M-MDSCs induced during EAE were found to potently suppress T cell activation through the production of NO. Furthermore, transfer of these activated cells led to T

cell apoptosis in the central nervous system and mitigated the EAE phenotype (59). The above data are not mutually exclusive. However, further research is needed in order to pick such a population that maximizes the immune suppression.

4. Induction of tolerance must take into consideration the timing of a given treatment. Several studies to date support MDSCs accumulation in autoimmune settings. However, disease progression is still evident indicating that endogenous MDSCs are rather ineffective at mitigating autoimmune disorder. Two possibilities that require further investigation could occur: either MDSCs do suppress autoreactive T cell proliferation but damaged tissue compounds still induce inflammatory response and expand tissue destruction or under chronic inflammation such cells reach a point at which they become inactivated enabling autoreactive cells to continue their damaging effect. Consequently, if an autoimmune disorder is identified early enough, MDSCs might be effective to control disease progression. Indeed, administration of exogenous MDSCs in certain mouse models such as alopecia areata (113) and a model of T1D (63) successfully reversed disease progression suggesting that MDSCs could be used as a cellular therapy for autoimmune diseases. Thus, efforts should focus in the identification of biomarkers that monitor host's immune status, type and stage of disease, so that the appropriate cell-based therapy is given at the right time possibly prior to the appearance or at the initial state of the disease before tissue damage and complications ensue.

5. Understanding the cross-talk between MDSCs and cells mediating the autoimmune pathogenesis. As already mentioned, MDSCs interact not only with T cells, by both diminishing their migration to activation sites and suppressing their proliferation, but also target other immune cells. Thus, MDSCs interact with NK cells by inhibiting their cytotoxic effect. Moreover, IL-10 produced by MDSCs promote the development of iTreg cells, which can suppress T cells by inhibiting their attachment and decrease IL-12 secretion by macrophages (114). Since we are just beginning to understand the complexity of this network, it is likely that there are additional interactions that further mediate immune response. An improved understanding of the co-dependency between MDSCs and other immune cells will aid in the search for adapting MDSCs as a therapeutic tool for autoimmune diseases.

5. OBJECTIVES

The present study sought to investigate the immunomodulatory properties of MDSCs in experimental inflammatory colitis and T cell-mediated immune responses from IBD patients. More specifically, the objectives of the study were:

1. **To dissect the contribution of MDSCs in the regulation of IBD.**
2. **To investigate BM-MDSCs therapeutic potential.**
3. **To understand how colitis milieu affects BM-MDSCs function.**
4. **To delineate the mechanism(s) underlying this process.**

6. MATERIALS AND METHODS

IBD patients

IBD patients were recruited through the Gastroenterology Department, University Hospital of Heraklion (Crete, Greece). The disease's diagnosis and classification was established by clinical criteria and endoscopic/histopathological findings of World Gastroenterology Organisation Global Guidelines. Disease activity was determined by Ulcerative Colitis Activity Index and Harvey-Bradshaw Crohn's disease activity index. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete) approved this study. Informed consent was obtained from all patients prior to sample collection.

Reagents

For human cell phenotypes, the following fluorescent-conjugated monoclonal antibodies were used: CD15 (8OH5), CD33 (D3HL60.251), CD14 (RM052), CD25 (B1.49.9) from Beckman Coulter, Fullerton, CA, HLA-DR (L243, G46-6) and CD4 (RPA-T4) from BD Pharmingen, San Diego, CA.

For analysis of mouse cells the following fluorescent-conjugated monoclonal antibodies were used: Ly6C (1G7.G10) from Miltenyi Biotec, Bergisch Gladbach, Germany, Gr-1 (RB6- 8C5) from eBioscience, San Diego, CA, CD11c (N418) from BioLegend, San Diego, CA, CD11b (M1/70), CD3e (145-2C11), CD19 (1D3), CD4 (RM4-5), Ly6G (1A8), CD44 (Pgp-1, Ly24), CD25 (PC61) from BD Pharmingen, San Diego, CA.

Cell cultures were performed in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640, both supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 2-Mercaptoethanol (2-ME) ($5x10^{-5}$ M) all from Gibco, Carlsbad, CA. 2,4,6-Trinitrobenzene Sulfonic Acid Solution (TNBS) was purchased from Sigma-Aldrich, St.Louis, MO.

Human mononuclear cell isolation from peripheral blood

Heparinized blood was collected from healthy subjects and IBD patients, and peripheral blood mononuclear cells (PBMCs) were isolated on Histopaque-1077 (Sigma-Aldrich, St.Louis, MO) density gradient. MDSCs and $CD4^+$ T cells were analyzed by flow cytometry and sorted as described. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete) approved this study.

Human intestinal mucosal mononuclear cell isolation

Lamina propria mononuclear cells **(**LPMCs) were isolated from intestinal biopsy specimens as previously described with slight modifications (115). Fresh dissected tissue was incubated in calcium and magnesium-free Hanks' Balanced Salt Soln (HBSS) (Sigma-Aldrich, St.Louis, MO) to remove mucus. The mucosa was then incubated twice in HBSS containing 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St.Louis, MO) for 60min at 37^0C. Tissues were collected and incubated in RPMI 1640 (Gibco, Carlsbad, CA) containing 0.5mg/ml collagenase D, 0.1mg/ml DNase I, and 1mg/ml hyaluronidase (Sigma-Aldrich, St.Louis, MO) for 10min on a shaker at 37^0C. The fraction was pelleted and mononuclear cells were purified on 30%/70% discontinuous Percoll gradient (Sigma-Aldrich, St.Louis, MO).

T cell *in vitro* suppression assay from IBD subjects

Human $CD4^+CD25^-$ T cells were sorted (purity >99%) from PBMCs, labeled with carboxyfluorescein succinimidyl (CFSE) (eBioscience, San Diego, CA) (1μM for 10min at 37^0C in labeling buffer-PBS/0.1% BSA) (Sigma-Aldrich, St.Louis, MO) and cocultured with autologous sorted $HLA\text{-}DR^{-/low}CD14^-CD15^+CD33^+$ cells (purity >95%) at 1:1 ratio, in the presence of 2μg/ml plate bound anti-CD3 (OKT) and 1μg/ml anti-CD28 (CD28.2), both purchased from eBioscience, San Diego, CA. Proliferation of T cells was determined based on CFSE dilution by flow cytometry.

Mice

Female Balb/c mice (8-10wk) were obtained from the specific pathogen-free facility of the Institute of Molecular Biology and Biotechnology (IMBB Heraklion, Crete, Greece). DO11.10 TCR transgenic (Tg) mice were kindly provided by Dr.Panoutsakopoulou (Biomedical Research Foundation of the Academy of Athens, Athens, Greece). All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office.

TNBS-induced colitis

Acute TNBS-induced colitis was induced by intrarectal injection of 2-3mg TNBS dissolved in 40% ethanol, as described previously (108). Mice were lightly anesthetized with diethyl ether and then slowly administered 100μl of TNBS solution via a 3.5-F catheter, equipped with a 1-ml syringe; the catheter was advanced into the rectum until the tip was 4 cm proximal to the anal verge. After the intrarectal injection, the mice were held in a high-tail position for 60sec to ensure that TNBS solution remains completely in the colon lumen. Mice were monitored daily for weight loss. Five days after TNBS delivery mice were killed. Spleens and mesenteric lymph nodes were removed and processed appropriately.

***In vivo* depletion of MDSCs**

To deplete $Gr1^+$ cells mice were injected intraperitoneally (i.p.) with 250μg of RB6-8C5 depleting mAb (Biolegend, San Diego, CA) on day 0 (day 0: TNBS instillation) and 250 μg of RB6-8C5 on day 2. Efficient depletion was confirmed by staining and flow cytometric analysis on homogenized spleen suspension. The percentage of live 7-Amino Actinomycin (7-AAD^-) (BD Pharmigen, San Diego, CA) $Gr1^+CD11b^+$ cells in spleen (gated on $CD3^-CD19^-$) was measured on day 5 after instillation.

Adoptive transfer experiments

For adoptive transfer experiments, either *in vitro* generated from naïve BM or *ex vivo* isolated from BM of murine colitis live $7AAD^{-}CD3^{-}CD19^{-}$ $CD11b^{high}Gr1^{+}$ cells were sorted (purity>95%) and transferred ($2x10^{6}$ cells) intravenously (i.v.) into syngeneic TNBS-instilled mice on days 0 and 2 after TNBS administration. Mice were monitored daily for clinical signs of disease.

Mouse lamina propria mononuclear cell isolation

LPMCs were isolated as described previously (116). Colons were cleared of fecal contents and incubated in calcium and magnesium free HBSS (Sigma-Aldrich, St.Louis, MO) for 10min. Mesenteric tissue and Peyer's patches were excised and discarded. Dissected colonic sections were then incubated containing 5mM EDTA (Sigma-Aldrich, St.Louis, MO) for 30min at 37^{0}C with gentle stirring to separate the epithelial compartment in the supernatant. Deepithelized colonic tissue was resuspended in HBSS with 0.5mg/ml collagenase D and 0.1mg/ml DNase I (Sigma-Aldrich, St.Louis, MO) for 60min at 37^{0}C. The fraction was pelleted and LPMCs were purified on a 30%/70% discontinuous Percoll gradient (Sigma-Aldrich, St.Louis, MO).

Histological analysis

To assess intestinal inflammation, mouse colons were dissected out, a piece of the proximal and one from the distal colon was fixed in 10% neutral buffered formalin, and embedded in paraffin. Transverse sections (6–7 μm) from colons were stained with Hematoxylin & Eosin (E&E), and analyzed in a blinded fashion for infiltrating cells using a Nikon Eclipse E800 microscope (Tokyo, Japan).

Flow cytometry and cell sorting

Single cell suspensions were prepared from tissues and cells were stained for extracellular markers for 20 min at 4 ℃ in 5% Fetal Calf Serum (FCS) (Gibco,

Carlsbad, CA) in phosphate-buffered saline (PBS) (Sigma-Aldrich, St.Louis, MO). Dead cells were identified and excluded from all analyses by 7AAD (BD Pharmingen, San Diego, CA). Cells were acquired on a FACS-Calibur (BD Biosciences, San Diego, CA) and analyzed using FlowJo software (Tree Star, San Carlos, CA). Cell sorting was performed using the high-speed cell sorter MoFlo (Dako, Santa Clara, CA).

Generation of $CD11c^{-}CD11b^{+}Gr1^{+}$ cells from bone marrow *in vitro*

MDSCs were generated as previously described (110). Tibias and femurs from Balb/c mice were removed and BM was flushed out. Red blood cells (RBCs) were lysed with RBC lysis buffer (Sigma-Aldrich, St.Louis, MO). To obtain BM-derived MDSCs, 2.5×10^{6} cells were plated into dishes with 100 mm diameter in 10 mL of complete medium supplemented with 30% supernatant from a murine GM-CSF-secreting X63Ag8 cell line (kindly provided by Dr. Eliopoulos AG, Molecular and Cellular Biology Laboratory, University of Crete Medical School, Heraklion, Greece). Cells were maintained at 37°C in 5% CO_2-humidified atmosphere. After 3 days, non-adherent cells were collected and $CD11c^{-}CD11b^{+}Gr1^{+}$ cells purified by sorting, while $Ly6G^{+}CD11b^{+}$ isolated using magneticbeads and MidiMACS, according to the manufacturer's instructions.

***In vitro* suppression assay**

Naïve mesenteric lymph nodes (MLNs) mouse $CD4^{+}CD25^{-}$ T cells were sorted (purity > 99%), labeled with CFSE (eBioscience, San Diego, CA) (1μM for 10min at 37^{0}C in labeling buffer 0.1% BSA in PBS) (Sigma-Aldrich, St.Louis, MO) and cocultured ($2x10^{4}$ cells/well) with sorted $CD11b^{+}Gr1^{+}$ BM-MDSCs (purity > 95%), at 1:1 ratio, in the presence of 2μg/ml plate bound anti-CD3 (145-2C11; BD Biosciences, San Diego, CA) and 1 μg/ml anti-CD28 (37.51; BD Biosciences, San Diego, CA). Activation of $CD4^{+}$T cells was determined based on CD25 expression, while proliferation based on CFSE dilution by flow cytometry.

In vivo suppression assay

Under the combined anesthesia with ketamine (Merial, Milano, Italy) and xylazine (Beyer, Leverkusen, Germany) spleens of naïve Balb/c mice were injected with and exposed to T cells mixed with equal numbers of antigen presenting cells (APCs), in the presence or absence of BM-MDSCs (at ratio 1:1). $CD4^+$ T cells enriched from spleen of DO11.10 Tg mice by sorting were labeled with CFSE. Dendritic cells (DCs) isolated from spleen of naïve Balb/c mice by CD3 depletion were primed *in vitro* with lipopolysaccharide (LPS) (0.25μg/ml; Invivogen, San Diego, CA) and loaded with ovalbumin (OVA) antigenic peptide (amino acids 323-339) (20μg/ml; Sigma-Aldrich, St.Louis, MO) for 2h at 37^0C. $Gr1^+CD11b^+$ BM-MDSCs were generated *in vitro* as described previously. On day 3, activation and proliferation of transferred $CD4^+$ T cell population were assessed based on CD44 expression and CFSE dilution, respectively by flow cytometry.

Phenotypic analysis

Sorted $Gr1^+CD11b^+$or magnetic bead isolated $Ly6G^+CD11b^+(7AAD^-CD3^-CD19^-)$ BM-MDSCs cells were cultured (1.5x 10^6cells/ml) in the presence of pooled sera either from naïve or TNBS treated mice (20μl/dish) for 24h. Cell surface markers were assessed by flow cytometry. Cultured supernatants assessed for production of IL-10 by ELISA (BD OptEIA^{tm}; BD Biosciences, San Diego, CA).

Western blot for CEBP*β* detection

Whole-cell lysates (40 mg protein) were subjected to SDS-PAGE electrophoresis on 10% gels and then transferred to nitrocellulose membranes (Protran; Whatman, GE Healthcare, Chalfont, St.Giles, UK). Membranes were blocked with 5% milk in Tris-Buffered Saline Tween-20 (TBST) and then incubated with anti-CEBP*β* (1:1000; Abcam, Cambridge, UK), as well as anti–actin (1:2000; Sigma-Aldrich, St.Louis, MO) as a loading control. Detection was performed by using HRP-conjugated anti-immunoglobulin (Sigma-Aldrich, St.Louis, MO) and chemiluminescent reagents (Supersignal Substrate; Pierce, Waltham, MA).

Statistics

Two-tailed Student's *t* tests were carried out and p values are reported. Mann–Whitney *U* test was used for the statistical analysis of human MDSCs. Kaplan-Meier survival analysis was used to examine the impact of cell transplantation on the survival of the mice. All analyses were performed using the GraphPad Prism (GraphPad Software, La Jolla, CA).

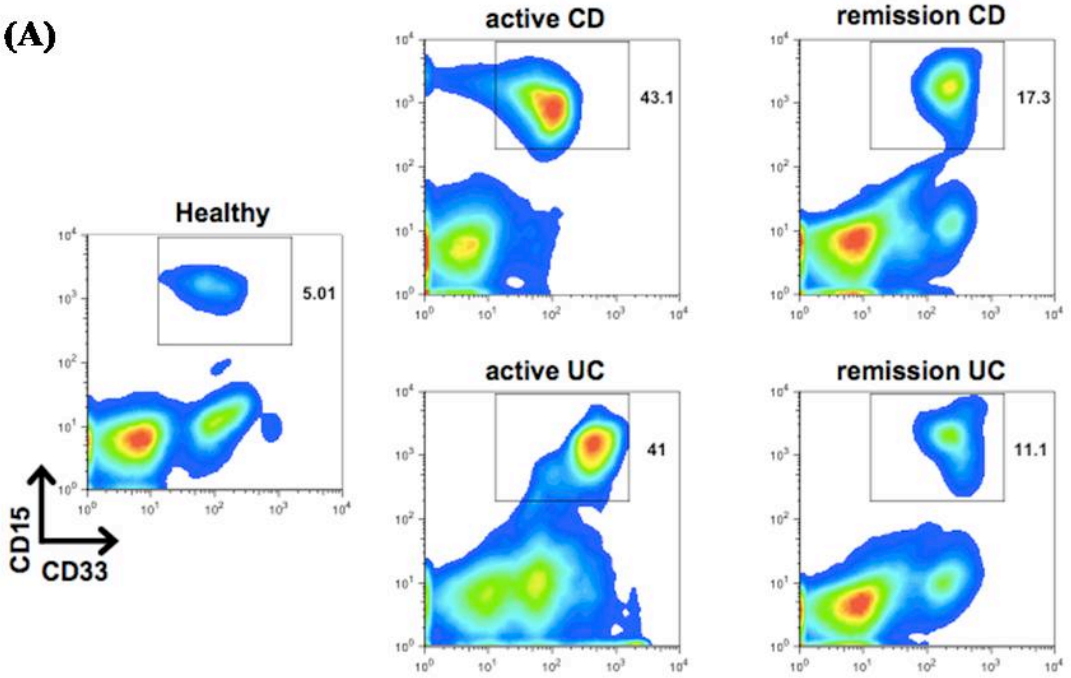
7. RESULTS

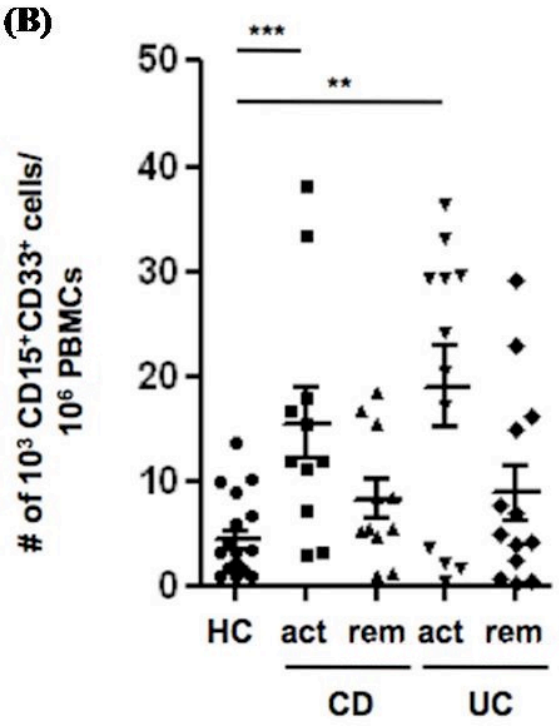
7.1. $CD33^{+}CD15^{+}$ MDSCs are enriched in the periphery of IBD patients

We initially screened IBD patients for the presence of MDSCs. Human MDSCs have mainly been described in patients with cancer and are characterized as $HLA\text{-}DR^{-/low}CD14^{-}CD33^{+}CD15^{+}$ cells (7) (60) (68). We stratified CD and UC patients into active and quiescent cohort and compared MDSCs of these groups to that of healthy controls (demographics in Table 1). Flow cytometry analysis demonstrated increased frequency (Figure 1A) and relative numbers (Figure 1B) of $HLA\text{-}DR^{-/low}CD14^{-}CD33^{+}CD15^{+}$ in both patients with active CD ($p=0.0008$) and UC ($p=0.009$) compared with healthy controls. In contrast, no elevation of MDSCs in patients with quiescent CD ($p=0.1$) or UC ($p=0.2$) was observed compared to the levels in healthy controls. However, within the CD ($p=0.1$) and UC ($p=0.08$) groups, no statistically significant difference was detected with respect to MDSCs levels (Figure 1B). Notably, looking at LPMCs of IBD patients, MDSCs were present only in CD lesions, when compared with biopsies either from UC or healthy control specimens (Fig.1C).

Table 1. Patients characteristics.

Type of disease	Stage of disease	No.	Male	Female	Average age (year)
Ulcerative colitis	Active Remission	12 13	19	6	47.9
Crohn's disease	Active Remission	11 11	14	8	45.3
Healthy		19	10	9	34.3


(A)
Healthy
5.01
active CD
43.1
remission CD
17.3
active UC
41
remission UC
11.1
CD15
CD33


(B)
***
**
# of 10³ CD15⁺CD33⁺ cells/ 10⁶ PBMCs
50
40
30
20
10
0
HC
act
rem
act
rem
CD
UC

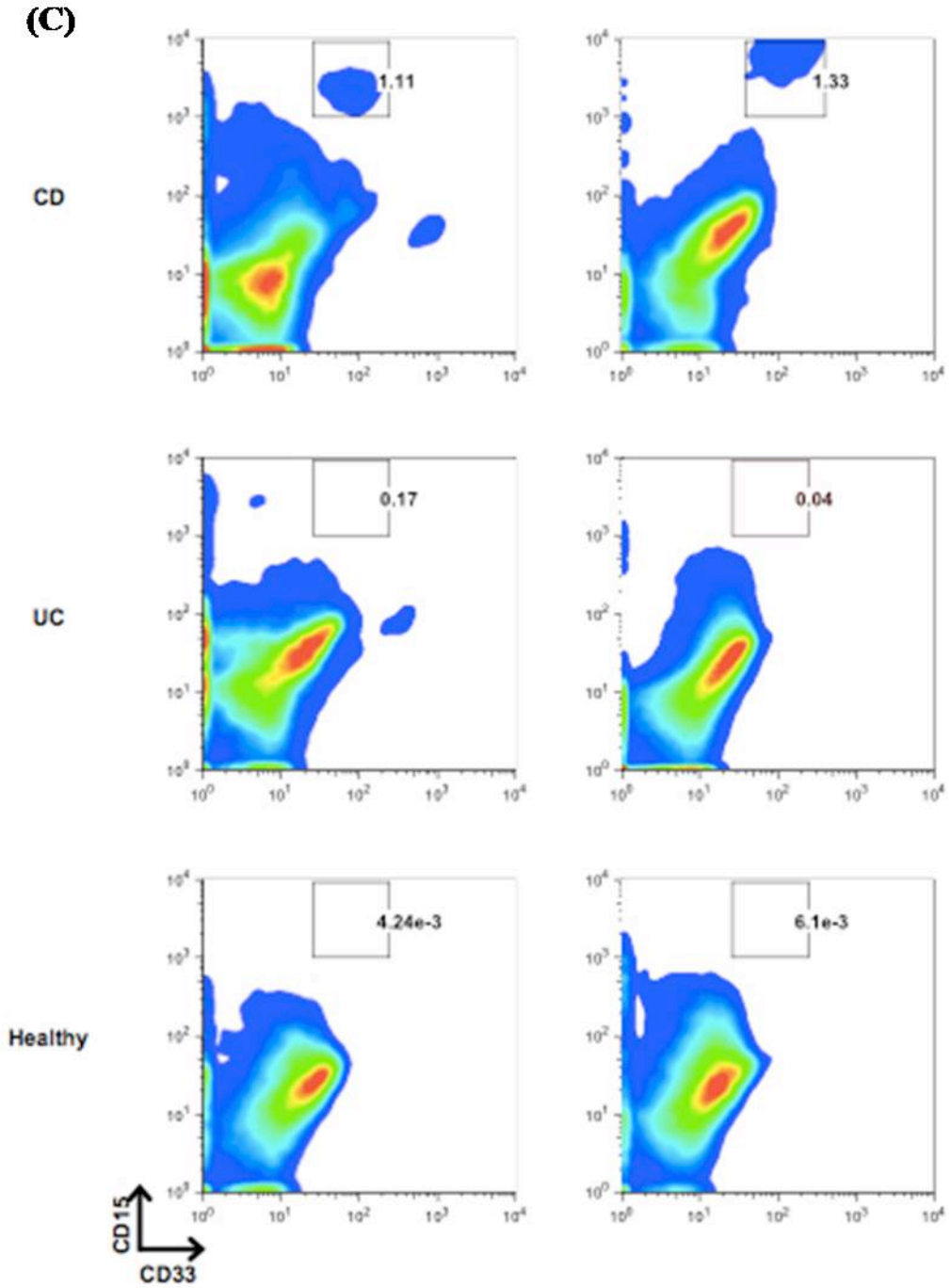
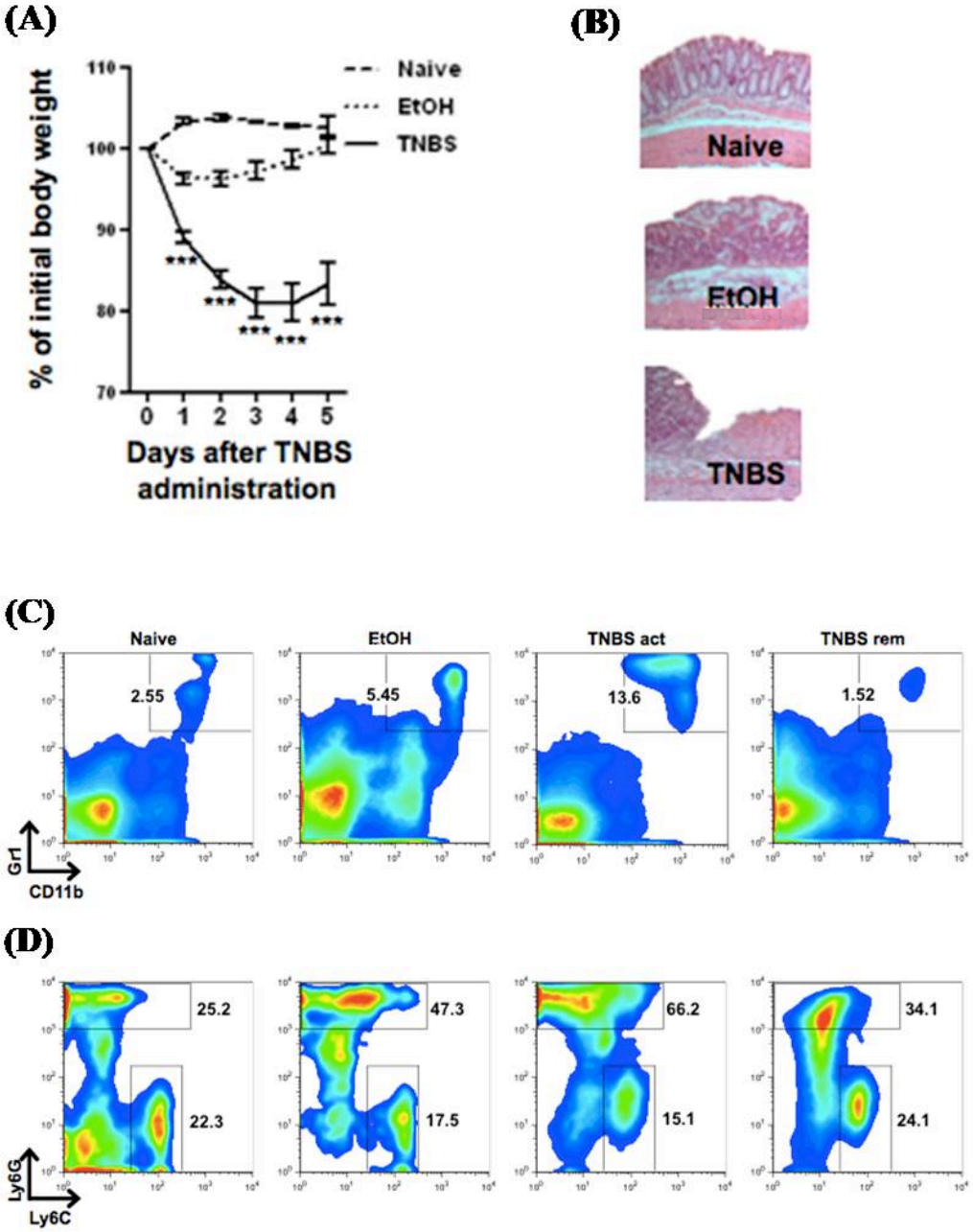


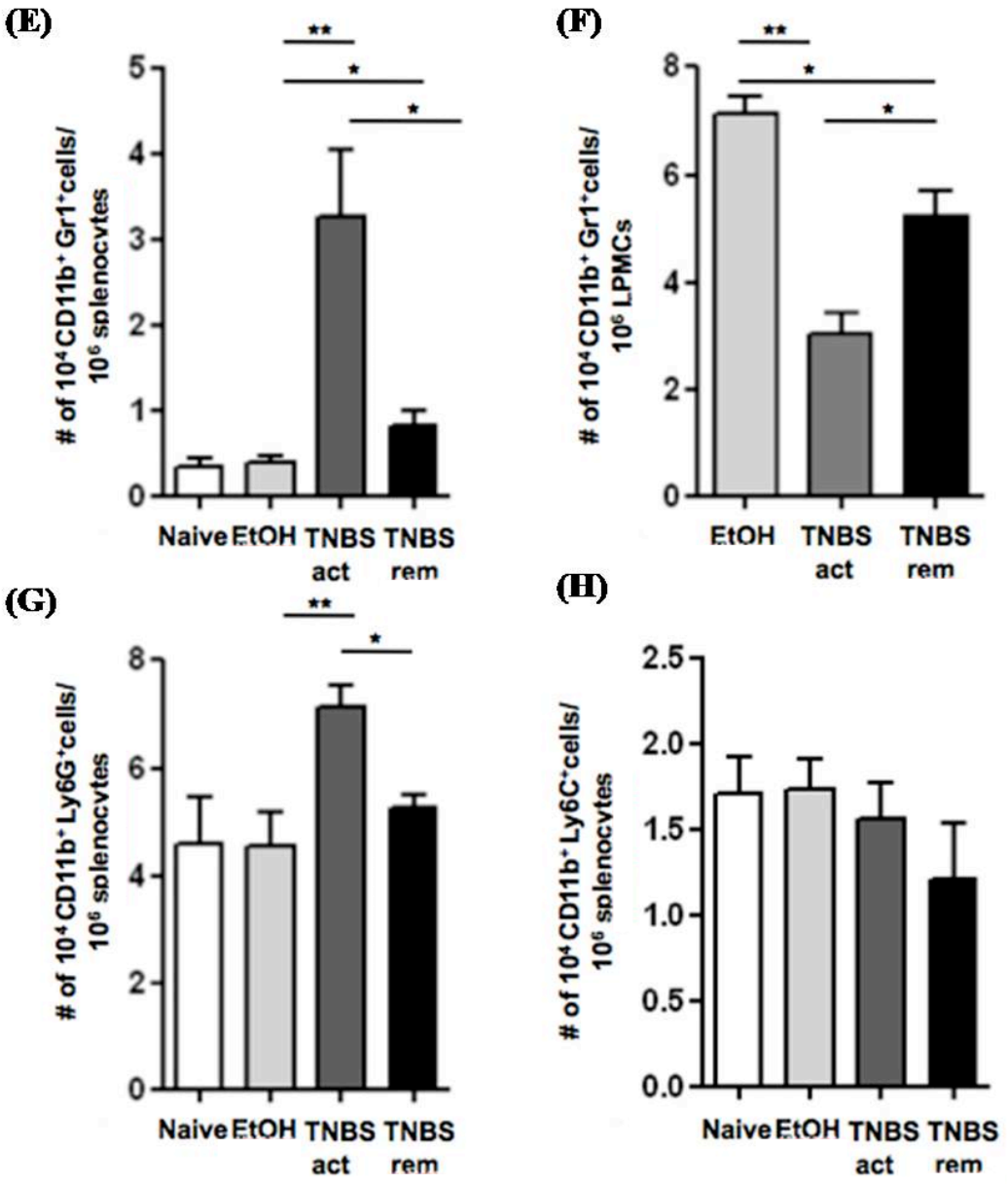
Figure 1. MDSCs are enriched in IBD patients. Frequency (A) and relative numbers (B) of $CD33^{+}CD15^{+}$ MDSCs in the peripheral blood of CD (n=22) and UC (n=25) patients with active disease or in remission, as well as healthy controls (n=19). Frequency (C) of $CD33^{+}CD15^{+}$ MDSCs in intestinal biopsies from patients with CD (n=2), UC (n=6) and healthy controls (n=7). Gates were set on $HLA\text{-}DR^{-/low}CD14^{-}$ $CD33^{+}CD15^{+}$ cells. Mann–Whitney *U* test *p* values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. HC, healthy controls; act, active; rem, remission.

7.2. MDSCs expand during TNBS colitis course

To delineate the role of MDSCs in intestinal inflammation, we used the TNBS mouse model of colitis, a commonly used animal model that resembles human Crohn's disease (108). Mice receiving TNBS developed severe colitis with an active phase characterized by bloody diarrhea and extensive weight loss until day 5, and followed by gradual weight recovery reflecting inflammation resolution (Figure 2A). Histological analysis showed that TNBS colitis was characterized by loss of architecture and marked inflammatory infiltration (Figure 2B). Using this mouse model, we assessed the expansion of $Gr1^+CD11b^+$ MDSCs in the spleen during the course of TNBS colitis. The frequency (Figure 2C), as well as the relative numbers (Figure 2E) of $Gr1^+CD11b^+$ MDSCs ($7AAD^-CD19^-CD3^-$) was significantly increased during the effector phase of colitis, while they contracted when mice entered remission. Interestingly, the relative numbers of $Gr1^+CD11b^+$ MDSCs were increased in colon LPMCs, during inflammation resolution (Figure 2F). In parallel, $CD11b^+$ cells present in the spleen of TNBS-treated mice were further analyzed for expression levels of Ly6G and Ly6C. Between the two subsets of MDSCs, only $Ly6G^+$ ($7AAD^-CD19^-CD3^-CD11b^+$) MDSCs resembled the expansion of $Gr1^+CD11b^+$ MDSCs during colitis course that is they were increased during the active phase and declined during inflammation resolution. In contrast, $Ly6C^+$ MDSCs were not significantly altered throughout the disease progress (Figure 2D, G, and H). These data demonstrated that TNBS-induced colitis is characterized by a rapid, but transient increase of MDSCs in the spleen, suggesting a potential role of these cells in the regulation of immune response.

To further investigate the contribution of MDSCs to TNBS-induced colitis, we depleted MDSCs through administration of the anti-Gr1 mAb to TNBS-treated mice on days 0 and 2. Although, anti-Gr1 mAb is not selective for depletion of MDSCs, since it could also deplete neutrophils, this is a commonly used strategy eliminating MDSCs in various murine models of cancer and autoimmunity (70, 72, 117). Interestingly, Gr1 depletion from the periphery of TNBS-treated mice rather aggravated the colitic phenotype (Figure 2I).


(A)
% of initial body weight
110
100
90
80
70
0 1 2 3 4 5
Days after TNBS administration
Naive
EtOH
TNBS
***
(B)
Naive
EtOH
TNBS
(C)
Naive
EtOH
TNBS act
TNBS rem
2.55
5.45
13.6
1.52
Gr1
CD11b
(D)
25.2
22.3
47.3
17.5
66.2
15.1
34.1
24.1
Ly6G
Ly6C


(E)
# of 10⁴ CD11b⁺ Gr1⁺ cells/ 10⁶ splenocytes
Naive
EtOH
TNBS act
TNBS rem
(F)
# of 10⁴ CD11b⁺ Gr1⁺ cells/ 10⁶ LPMCs
EtOH
TNBS act
TNBS rem
(G)
# of 10⁴ CD11b⁺ Ly6G⁺ cells/ 10⁶ splenocytes
Naive
EtOH
TNBS act
TNBS rem
(H)
# of 10⁴ CD11b⁺ Ly6C⁺ cells/ 10⁶ splenocytes
Naive
EtOH
TNBS act
TNBS rem

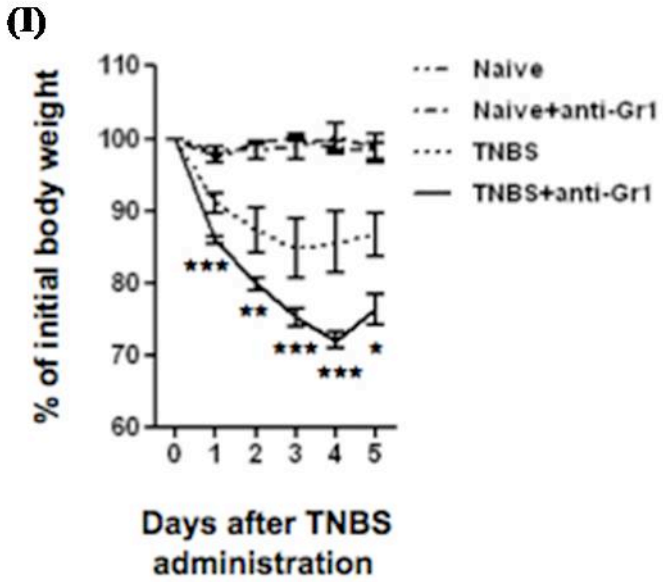


Figure 2. The role of MDSCs in TNBS-induced colitis. Mice were intrarectally administered 2–3 mg TNBS per mouse on day 0. (A) Body weight changes after TNBS instillation, and (B) H&E staining of colonic sections (10). $CD11b^{+}Gr1^{+}$ MDSCs (C) and Ly6G/C-MDSCs (D) accumulation in the spleen of mice during TNBS colitis. Representative flow cytometric analysis indicates percentages of MDSCs. Gates were set on $7AAD^{-}CD19^{-}CD3^{-}$ or $7AAD^{-}CD19^{-}CD3^{-}CD11b^{+}$ cells for C and D, respectively. Relative numbers of $CD11b^{+}Gr1^{+}$ MDSCs/10^{6} splenocytes (E) and $CD11b^{+}Gr1^{+}$ MDSCs/10^{6} LPMCs (F), $Ly6G^{+}$ (G), and $Ly6C^{+}$ (H) MDSCs/10^{6} splenocytes during the different phases of the disease. Data are shown as mean ± SD (n=3–6 mice/group). (I) Mice received 250 mg of anti-Gr1 mAb (RB6-8C5) i.p. on days 0 and 2. TNBS colitis was induced on day 0. Body weight of mice subjected to TNBS colitis treated with anti-Gr1 mAb. Data shown represent pooled values from four independent experiments. *T* test *p* values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. act, active; rem, remission.

7.3. BM-MDSCs exert suppressive function under steady state

To understand whether the colitis milieu affects MDSCs function, we utilized a protocol for *in vitro* generation of BM-MDSCs, as described by Rossner *et al*. (110). To this end, MDSCs were harvested as non-adherent cells from BM cell cultures supplemented with GM-CSF. Indeed, culture of BM cells with high doses of GM-CSF evoked efficient enrichment of the $Gr1^{+}CD11b^{+}$ population *in vitro* as depicted

in (Figure 3A). Although enriched *in vitro*, it remained to be determined whether BM derived $Gr1^{+}CD11b^{+}$ cells, could exert suppressive function. To address this, sorted BM-Gr1CD11b cells – enriched by GM-CSF – were co-cultured with autologous, CFSE-labeled MLN cells stimulated with anti-CD3/CD28. After 5d of coculture, BM-$Gr1^{+}CD11b^{+}$ cells potently suppressed activation (Figure 3B) and proliferation of responder $CD4^{+}CD25^{-}$ T cells in a dose dependent manner (Figure 3C).

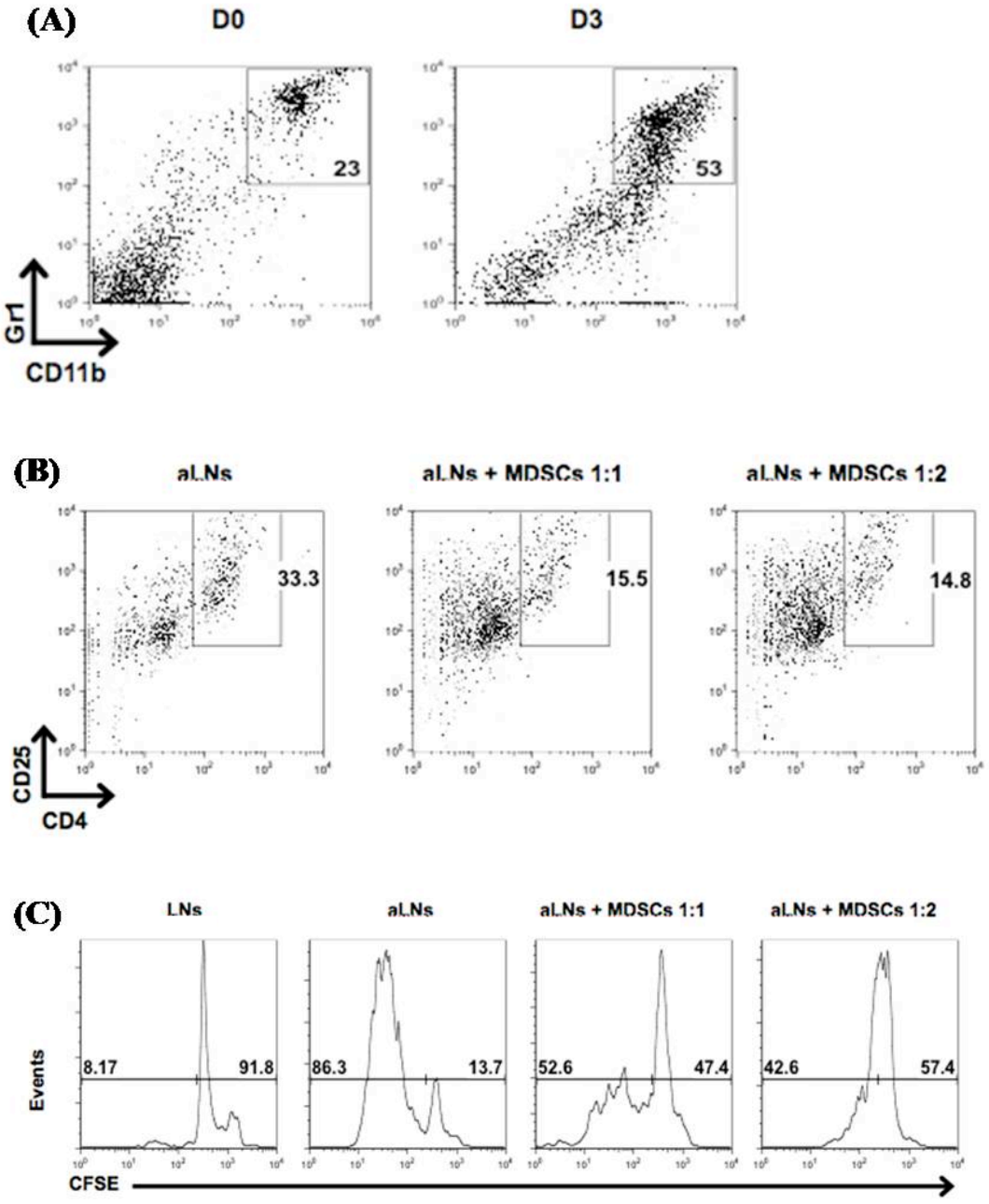
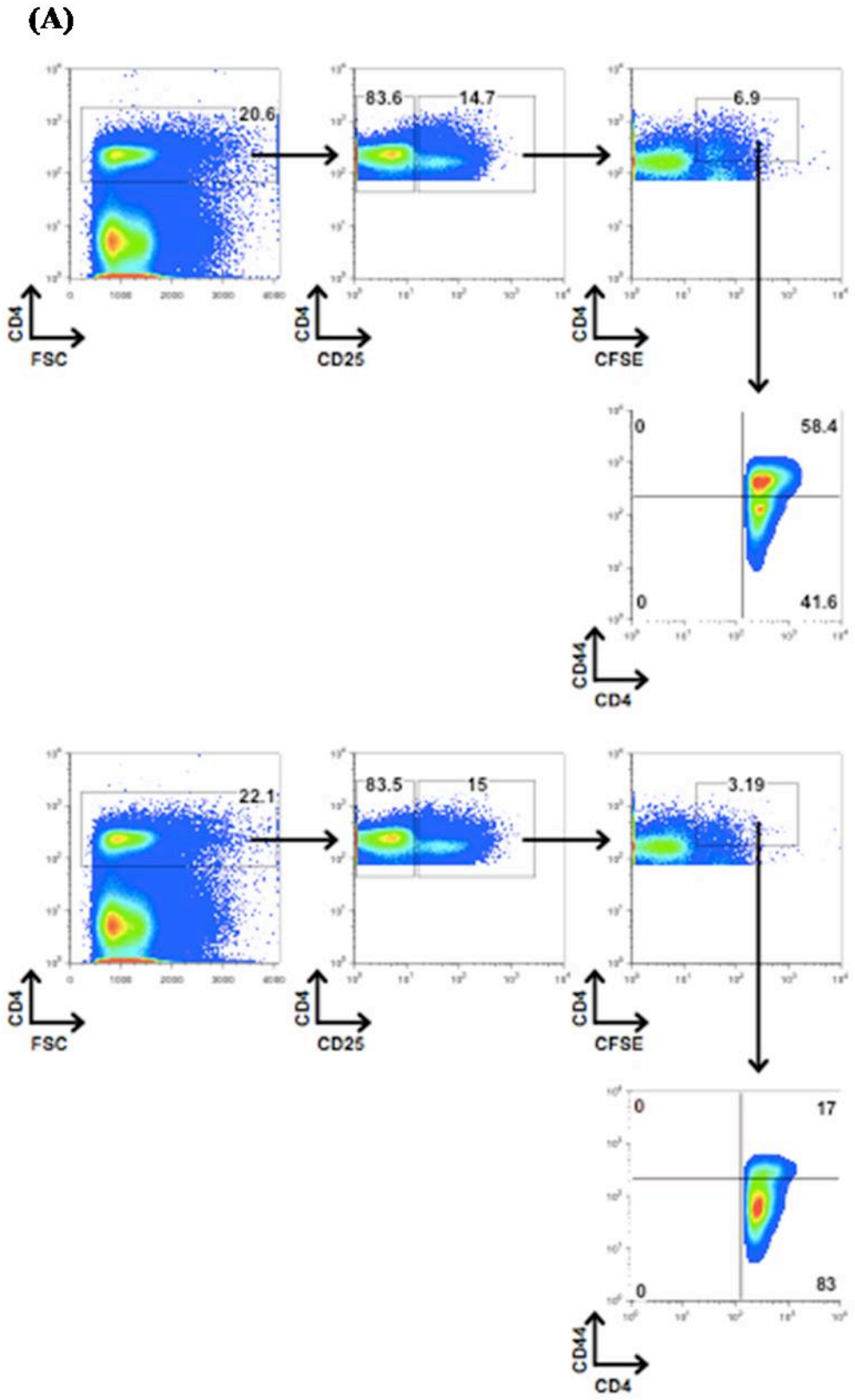


Figure 3. *In vitro* enrichment of BM-MDSCs. Culture of BM cells with high doses of GM-CSF for 3d evoked efficient enrichment of the $Gr1^+CD11b^+$ population *in vitro* (A). Sorted $Gr1^+CD11b^+$ BM-MDSCs were cocultured with CFSE-labeled MLN cells, activated by plate bound anti-CD3 and soluble anti-CD28. $CD4^+$ T cell activation and proliferation was measured by flow cytometry on day 5. Dot plots show CD4 versus CD25 on gated viable $CD4^+$ T cells (B). CFSE dilution of $CD4^+$ T cells. Numbers indicate the percentages of cells that proliferated (C). Results are representative of three independent experiments.

To investigate whether BM-MDSCs could suppress immune responses *in vivo*, naïve Balb/c mice were intrasplenically injected with CFSE-labeled DO11.10 T cells, ovalbumin-pulsed splenic dendritic cells, denoted as DC.OVA, and/or $CD11b^+Gr1^+$ MDSCs harvested from BM. At 72 h, activation and proliferation of $CD4^+$ T cells were analyzed using flow cytometry. Mice treated with DC.OVA plus BM-MDSCs exerted decreased proliferation and activation (Figure 4A, lower panel) compared to mice treated with DC.OVA alone (Figure 4A, upper panel), as indicated by CFSE dilution and CD44 – another T cell activation marker instead of CD25 – expression, respectively. MDSC-mediated suppression of T cell proliferation and activation was significant, as extrapolated by enumeration of the $CD4^+$ T cells (Figure 4B). Collectively, these data suggest that under steady-state BM-MDSCs suppress T cell activation and proliferation both *in vitro* and *in vivo*.


(A)
20.6
CD4
FSC
83.6
14.7
CD4
CD25
6.9
CD4
CFSE
0
58.4
0
41.6
CD44
CD4
22.1
CD4
FSC
83.5
15
CD4
CD25
3.19
CD4
CFSE
0
17
0
83
CD44
CD4

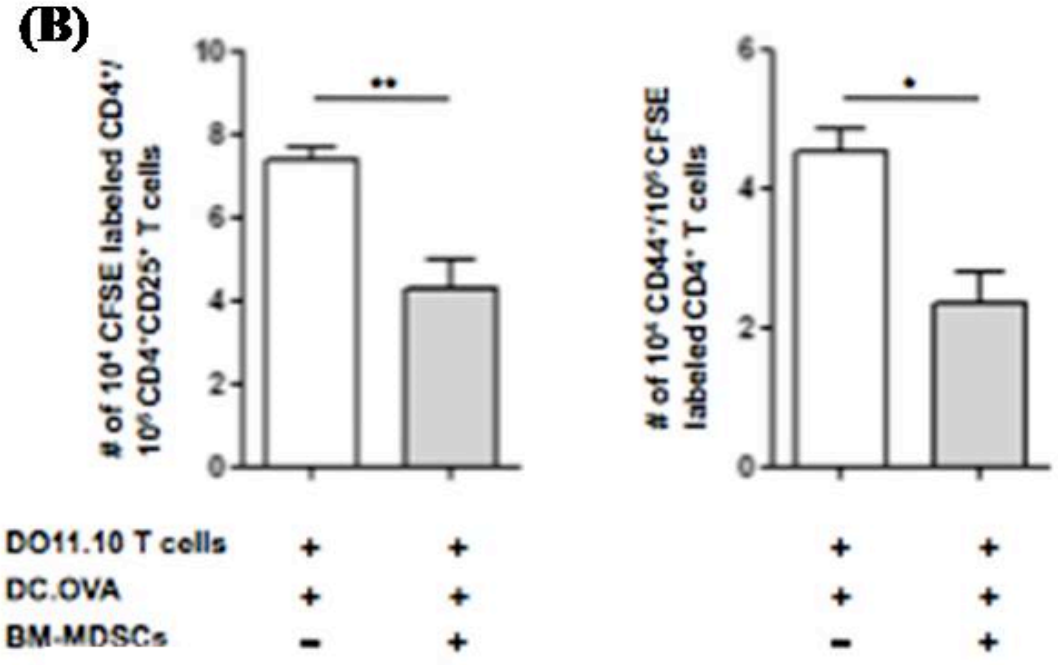


Figure 4. BM-MDSCs suppress T cell responses under steady state *in vivo*. (A) CFSE-labeled DO11.10 T cells (5 x 10^4) and DC.OVA (5 x 10^4) were intrasplenically injected into naïve Balb/c mice alone (upper panel) or with BM-MDSCs (5 x 10^4) (lower panel). On day 3, flow cytometry was performed. $CD4^+CD25^+$ T cells were gated and the fraction of CFSE-labeled $CD4^+$ T cells was determined. In the same experiment, CFSE-labeled $CD4^+$ T cells were analyzed for the expression of CD44. Experiments were performed with two mice/groups. (B) Relative numbers of CFSE-labeled $CD4^+$ T cells/10^6 $CD4^+CD25^+$ T cells (left panel) and $CD44^+$ T cells/10^6 CFSE-labeled $CD4^+$ T cells (right panel). Data are shown as mean ± SD (n=3–5 mice/group). *T* test *p* values: *, $p<0.05$; **, $p<0.01$.

7.4. BM-MDSCs fail to suppress ongoing colitis *in vivo*

To determine whether BM-MDSCs sustain their suppressive function *in vivo* under inflammatory conditions, highly pure BM-derived $Gr1^+CD11b^+$ MDSCs were adoptively transferred in TNBS-treated mice. Surprisingly, adoptive transfer of two doses of BM-MDSCs did not protect against colitis, but rather augmented the colitic phenotype, as indicated by the increased mortality rate (Figure 5A) and weight loss (Figure 5B) compared with TNBS only-treated mice. Furthermore, adoptive transfer of MDSCs isolated from the BM of TNBS-instilled mice into syngeneic hosts with

ongoing colitis, failed to regulate disease (Figure 5C). These data suggest that the inflammatory environment of TNBS colitis switched off the suppressive capacity of BM-MDSCs.

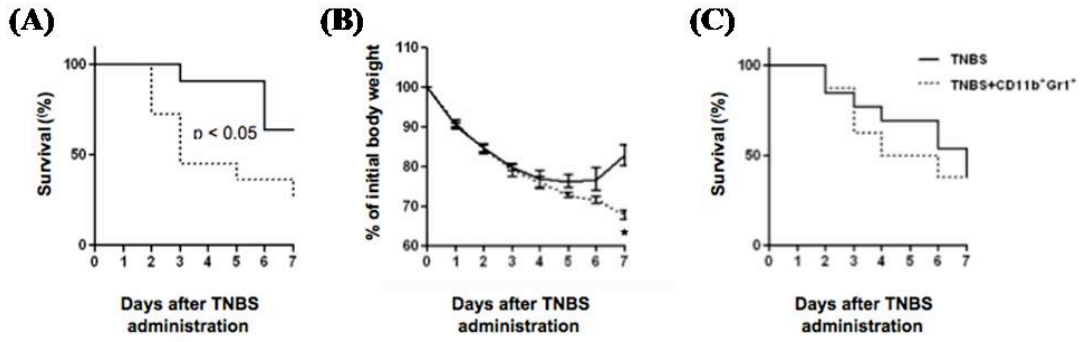


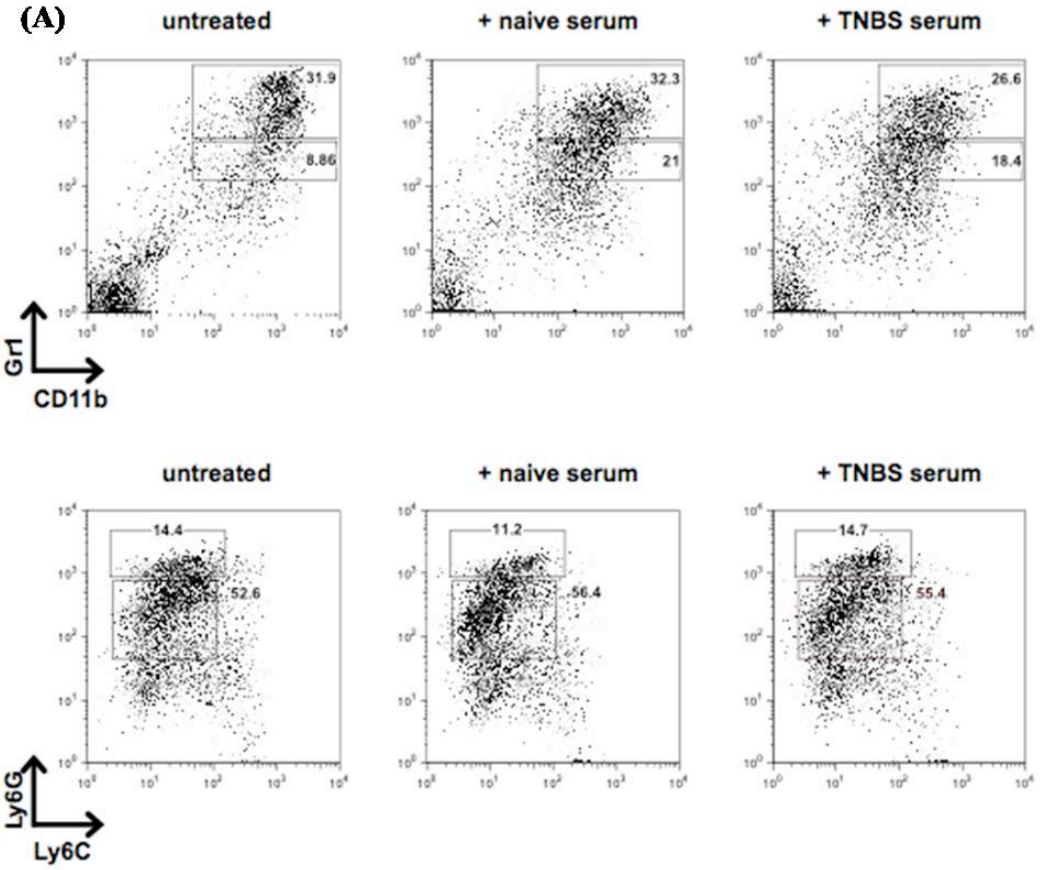
Figure 5. BM-MDSCs transfer aggravates TNBS colitis. TNBS-treated mice were i.v. injected with sorted *in vitro* generated $Gr1^+CD11b^+$ BM-MDSCs (2 x 10^6/mouse, purity>90%) on days 0 and 2 after TNBS administration. Mortality rate shown as Kaplan–Meier analysis (A) and body weight loss (B) of $Gr1^+CD11b^+$ BM-MDSCs treated and control mice. Data are shown as mean ± SD (n=11 mice/group). TNBS-treated mice were i.v. injected with sorted *ex vivo* derived $Gr1^+CD11b^+$ cells (2 x 10^6/mouse, purity>90%) isolated from BM of TNBS-instilled mice with ongoing disease on days 0 and 2 after TNBS administration. Mortality rate shown as Kaplan–Meier analysis (C) of $Gr1^+CD11b^+$ treated and control mice. Combined results of two independent experiments are shown (n=8–13 mice/group). *T* test *p* values: *, $p<0.05$.

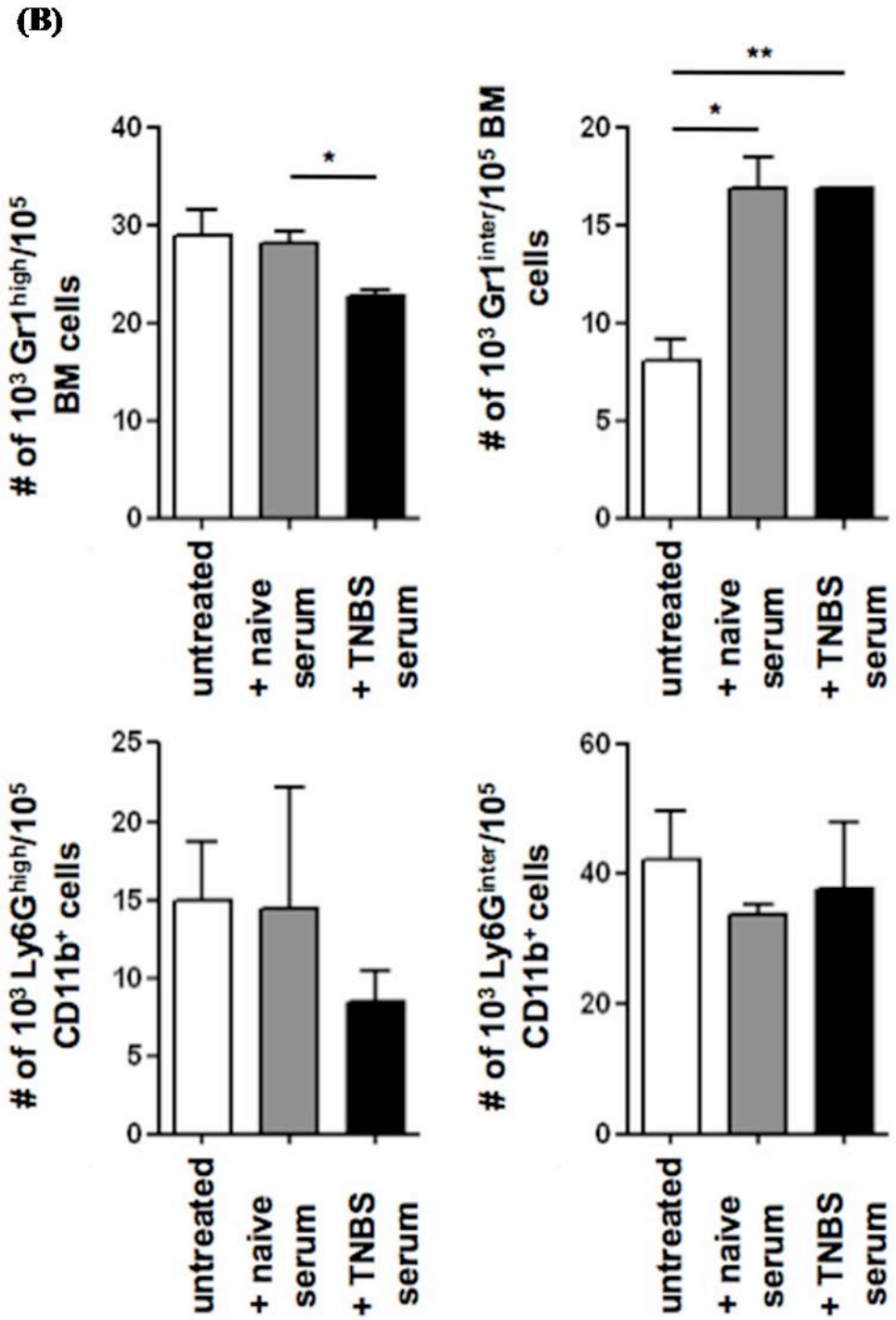
7.5. Characterization of BM-MDSCs in colitic environment

To explore the mechanism by which BM-MDSCs lost their suppressive properties in the TNBS colitis milieu, fresh BM cells, enriched by GM-CSF, were cultured in the presence of serum from either naïve or TNBS-treated mice. On day 3, cells were analyzed by flow cytometry. Importantly, serum from TNBS-treated mice led to a decreased proportion of $CD11b^+Gr1^{high}$, accompanied by increased relative numbers of $CD11b^+Gr1^{inter}$ cells (Figure 6A). Looking on $Ly6G^+$ no such differences were observed (Figure 6B). Furthermore, BM-MDSCs exposed to TNBS serum produced significantly lower levels of IL-10, a key anti-inflammatory cytokine, compared with untreated BM-MDSCs (Figure 6C).

Recent data suggest that CEBPβ controls the immunosuppressive function of MDSCs, linking the absence of CEBPβ transcriptional regulation to the loss of suppressive

activity (30). In accordance with this study, CEBPβ was barely detectable in freshly isolated $Gr1^{+}CD11b^{+}$, $CD11b^{+}Ly6G^{high}$ or $CD11b^{+}Ly6G^{low}$ BM cells. Interestingly, with the exception of $CD11b^{+}Ly6G^{high}$ cells, neither of the subsets of *in vitro* generated BM-MDSCs expressed CEBPβ. Even in the case of MDSCs isolated from BM of TNBS-treated mice, CEBPβ could not be detected, further supporting the idea that BM-MDSCs lose their suppressive properties under the specific inflammatory environment (Figure 6D).




(B)
# of 10³ Gr1high/10⁵ BM cells
# of 10³ Gr1inter/10⁵ BM cells
# of 10³ Ly6Ghigh/10⁵ CD11b⁺ cells
# of 10³ Ly6Ginter/10⁵ CD11b⁺ cells
untreated
+ naive serum
+ TNBS serum
*
**

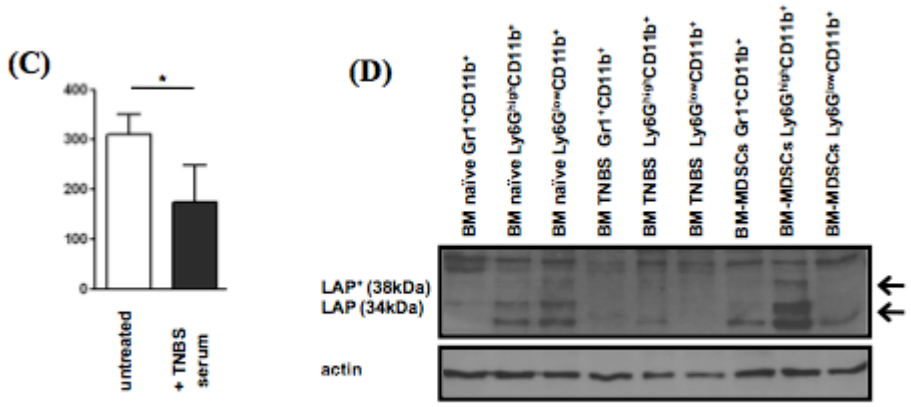


Figure 6. Phenotypic characterization of BM-MDSCs in colitic environment. Freshly isolated BM cells were cultured with GM-CSF in the presence of serum harvested either from naïve or active colitic mice. Untreated BM-MDSCs were used as controls. On day 3, frequency and relative numbers (mean ± SD) of $Gr1^{high}CD11b^{+}$, $Gr1^{low}CD11b^{+}$ (A), $Ly6G^{high}$, $Ly6G^{low}$ BM-MDSCs (B) were assessed by using flow cytometry. Gates were set on $7AAD^{-}CD19^{-}CD3^{-}CD11c^{-}$ and $7AAD^{-}CD19^{-}CD3^{-}CD11b^{+}$ cells. (C) Supernatants were assessed for IL-10 production by ELISA. Data are representative of three independent experiments. *T* test *p* values: *, $p<0.05$; **, $p<0.01$. (D) CEBP*β* expression was assessed by western blot analysis in sorted BM-derived $Gr1^{+}CD11b^{+}$, $Ly6G^{high}$, and $Ly6G^{low}$ cells either from naïve or TNBS-treated mice and *in vitro* generated BM $Gr1^{+}CD11b^{+}$, $Ly6G^{high}$, and $Ly6G^{low}$ cells. Detectable CEBP*β* isoforms are LAP*=38 KD and LAP=34 KD, as indicated. Actin was used as a loading control. Representative blot from four independent experiments.

7.6. Human MDSCs promote T cell proliferation in IBD patients

We next evaluated the suppressive ability of $CD33^{+}CD15^{+}$ MDSCs from patients with IBD. To this end, purified MDSCs ($CD33^{+}CD15^{+}HLA\text{-}DR^{-/low}CD14^{-}$) (Figure 7B) from peripheral blood of IBD patients were added to autologous sorted CFSE-labeled $CD4^{+}CD25^{-}$ T cells (Figure 7A), stimulated with anti-CD3/CD28 and proliferation was analyzed on day 5. Surprisingly, $CD33^{+}CD15^{+}$ MDSCs not only failed to suppress, but rather promoted T cell proliferation, as indicated by CFSE dilution

(Figure 7C). Notably, the percentage (Figure 7D) and total numbers (Figure 7E) of undivided $CD4^+$ T cells is diminished upon addition of MDSCs in the culture, suggesting that MDSCs markedly promoted the proliferation of T cells. Collectively, these results highlight the important role of MDSCs in IBD course, but also raise an intriguing question concerning their function.

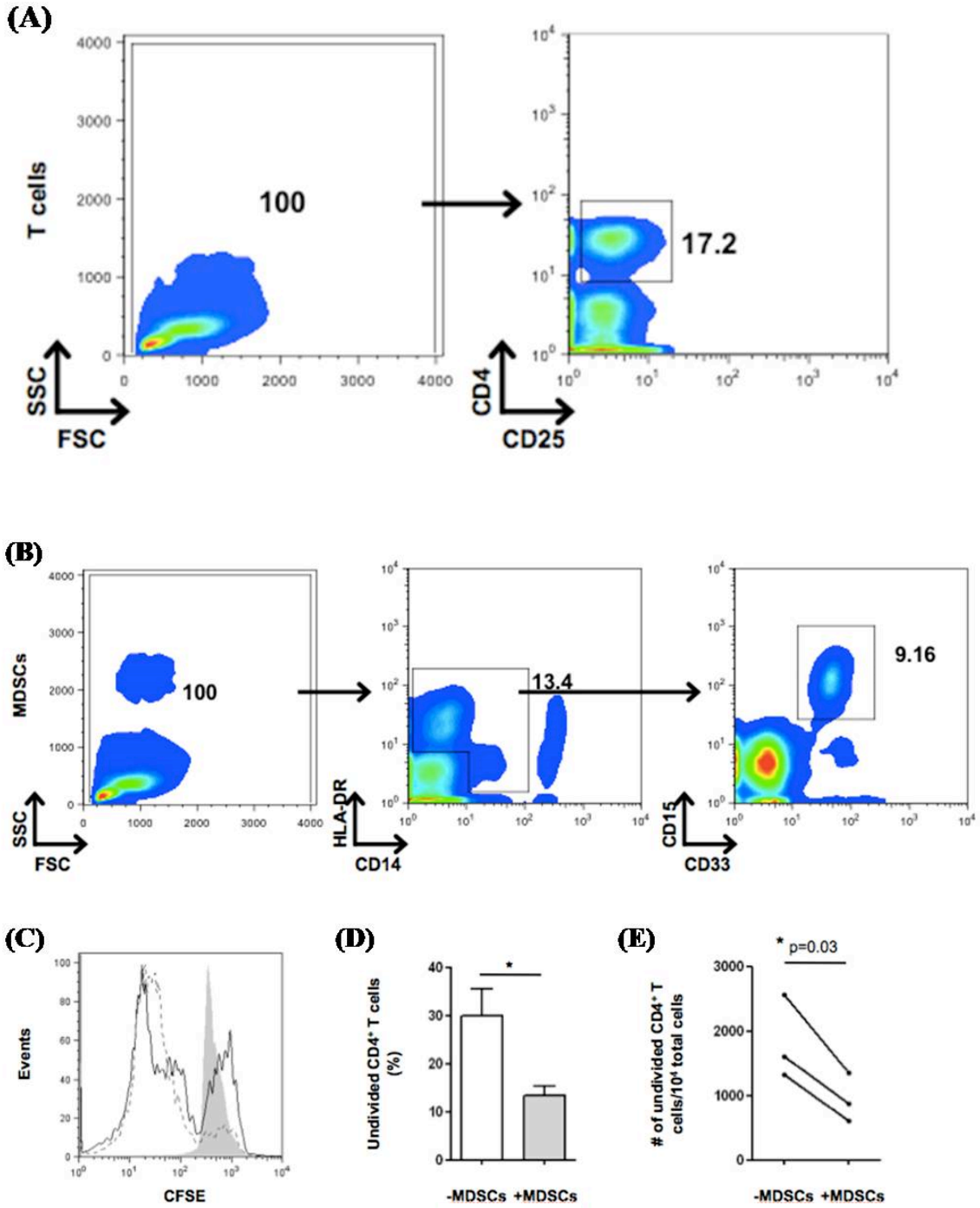


Figure 7. Human MDSCs promote T cell proliferation in IBD patients. FACS plots demonstrate the gating strategy used to isolate T cells (A) and MDSCs (B) from peripheral blood of IBD patients. (C) Sorted $CD33^{+}CD15^{+}$ MDSCs from IBD patients with active disease were cocultured with autologous $CD4^{+}CD25^{-}$ T cells stimulated with plate-bound anti-CD3 (1 mg/ml) and anti-CD28 (1 mg/ml) at 1:1 ratio for 5 d. T cell proliferation was measured as CFSE dilution by flow cytometry. $CD4^{+}$ T cells alone (grey fill), anti-CD3/CD28 activated $CD4^{+}$ T cells (solid line), anti-CD3/CD28 activated $CD4^{+}$ T cells plus $CD33^{+}CD15^{+}$ MDSCs (dashed line). CFSE dilution graph is representative of three independent experiments. (D) The percentage and (E) total numbers of undivided $CD4^{+}CD25^{-}$ T cells in the presence or absence of MDSCs in the culture is shown. Paired *T* test *p* values: *, $p<0.05$.

8. DISCUSSION

Restoration of immune homeostasis and self-tolerance represents the ultimate goal in IBD therapy. A comprehensive understanding of the interactions between several immune cells contributing to the intestinal inflammation should provide insights into the design of immunotherapeutic interventions. In this study, we show that the frequency of MDSCs increased dramatically in the periphery of IBD patients upon exacerbation of disease. Interestingly, although amplified, these cells failed to suppress autologous T cell proliferation *in vitro*. Accordingly, mouse studies demonstrate an expansion of MDSCs, particularly the granulocytic subset, in the peripheral lymphoid compartment, during active colitis. Importantly, *ex vivo* expanded BM-MDSCs, which potently suppress T cell responses under steady state, failed to abrogate the colitic phenotype, when adoptively transferred to TNBS-treated mice. Under such an inflammatory environment, BM-MDSCs switched phenotype and downregulated CEBPβ expression, a master regulator of the immunosuppressive function of MDSCs. Together, our results indicate that although active colitis induces recruitment of MDSCs, they are not competent to effectively limit autoimmune T cell responses.

It is rather difficult to demonstrate whether MDSCs described in this article represent a subset of neutrophils that have regulatory functions under pathologic conditions or a distinct myeloid cell population. Although granulocytic MDSCs and ‘‘regular’’ neutrophils share similar phenotypic traits, it has been proposed that they also possess important differences. G-MDSCs could be distinguished from neutrophils based on the morphology of the nucleus, with the former having ring-shaped appearance indicative of their immature state but mainly on their *in vitro* suppressive activity (68). Recently, whole-transcriptome analysis revealed a clear difference between G-MDSCs from tumor-bearing mice and neutrophils from tumor-free mice. G-MDSCs have an elevated expression of genes involved in the cell cycle, autophagy, G-protein signaling, and CREB pathway, whereas neutrophils have a higher expression of genes associated with NF-kB signaling and lymphotoxin-β receptor signaling. Further analysis confirmed that neutrophils have substantial higher basal levels of phosporylated c-Jun, p38, JNK, and ERK1/2 than G-MDSCs. Activated neutrophils express significantly higher level of TNF-α as compared to G-MDSCs (118). Morevover, genes encoding chemokines and their receptors associated with migration

are differentially expressed in G-MDSCs and neutrophils. Neutrophils have increased levels of CXCL4 and CXCL12 and reduced expression of CCL3, CCL4 and CXCL2 as compared to G-MDSCs (119). On the other hand, G-MDSCs express lower levels of CXCR1 and CXCR2, which are necessary for neutrophil extravasation from the bloodstream and subsequent tissue infiltration (120). However, this issue has not been addressed in this study and warrants further investigation.

Thus far, MDSCs have been extensively studied in patients with different types of tumors, but rarely examined in patients with autoimmune diseases. In human IBD, MDSCs are even less characterized. Haile *et al*. found that mononuclear HLA-DR$^{-/low}$CD14^{+} cells were elevated in IBD patients compared with healthy controls, while coculturing of these cells *in vitro* with autologous stimulated PBMCs resulted in a dose dependent suppression of proliferation and cytokine production (79). Subsequently, Xi *et al*. confirmed the elevation of HLA-DR$^{-/low}$CD14^{+} cells in periphery of IBD patients, but also associated the exacerbation of IBD with higher levels of MDSCs (87). In our study, we demonstrate that MDSCs were significantly enriched in the periphery of IBD patients and importantly, they not only failed to suppress but rather promoted responding autologous stimulated T cell proliferation. Notably, for the first time to our knowledge, here we show that MDSC-like cells were also present in biopsies from CD patients compared to both healthy controls and UC patients. However, the sample size was rather small to allow statistical analysis and further functional characterization of the isolated cells. In contrast to the above studies, our results refer to granulocytic MDSCs defined as HLA-DR$^{-/low}$CD14^{-} CD33^{+}CD15^{+} cells, possibly indicating that the two subsets exert different functionality in autoimmune responses.

In experimental IBD, MDSCs were first described in a CD8^{+} T cell-mediated model of colitis. The frequency of CD11b^{+}Gr1^{+} MDSCs with immunosuppressive function was increased in the spleen and intestine of colitic mice. Furthermore, cotransfer of transgenic T cells with MDSCs ameliorated intestinal inflammation (79). More recently, murine data were confirmed in chronic DSS-, as well as, in TNBS-induced colitis. An increase of MDSCs in the spleen and/or intestine correlated with the severity of intestinal inflammation, while adoptive transfer of either splenic or *in vitro* generated MDSCs ameliorated enterocolitis (79) (80) (81) (82). Consistent with the above studies, our data support an increase of CD11b^{+}Gr1^{+} MDSCs, and more specifically Ly6G^{+} subset, in the spleen of TNBS-treated mice. However, their

percentage in colon LPMCs during active TNBS-induced inflammation was rather decreased. More importantly, adoptive transfer of either *in vitro* generated $CD11b^+Gr1^+$ MDSCs or MDSCs isolated from BM of TNBS-treated mice into syngeneic hosts with ongoing colitis further augmented colitic phenotype. Although, surprising at first sight, this result is in line with studies supporting a proinflammatory role of MDSCs in experimental colitis, as they differentiate into inflammatory dendritic cells and macrophages (84) (85) or function as APC cells, triggering T cell proliferation (86). The disparity in these results could be attributed to the functional plasticity of the heterogenous population of MDSCs, which may be dictated by the inflammatory environment and the disease stage. In support of this, it is worth mentioning here the difference between the animal models used to mimic IBD and explore MDSCs efficacy. Guan *et al*. used a more chronic model of colitis with administration of TNBS twice over 1-week interval (81). By contrast, using an acute model in our study, we speculate that T cells, assumed to be the primary target of MDSCs, may not be fully engaged to suppressive signals by MDSCs.

Several lines of evidence have supported that MDSCs exert their suppressive function using a number of mechanisms. Recent data suggested that the molecular program underlying the immunoregulatory properties of MDSCs depends on CEBP*β*. Marigo *et al*. (30) showed that lack of CEBP*β* abrogated the suppressive capacity of both murine and human *in vitro* generated BM-MDSCs, at least through the reduction in arginase 1 and nitric oxide 2 proteins, while it did not affect their ability to proliferate. Consistent with this study, our results demonstrated that CEBP*β* was barely detectable in freshly isolated $Gr1^+CD11b^+$, $CD11b^+Ly6G^+$ or $CD11b^+Ly6G^{low}$ BM cells, lacking inhibitory function. Notably, none of these cell populations isolated from TNBS-treated mice expressed CEBP*β* isoforms. This finding may provide an explanation why MDSCs, although accumulated in the colitic inflammatory milieu, failed to effectively control aberrant T cell responses. Further evidence support that CEBP*β* expression in myeloid progenitor cells is directly regulated by STAT3. STAT3 sustains c-myc transcription and thus proliferation of immature granulocytes by direct binding to the c-myc promoter as well as enhancing CEBP*β* interaction at the c-myc promoter elements through control of CEBP*β* protein levels (31). Interestingly, a recent study shed light on immunoregulatory mechanisms of STAT3 during intestinal inflammation. Myeloid cell specific STAT3 hyperactivation in a mouse model of DSS-induced colitis resulted in reduced disease severity and amelioration of intestinal

inflammation through expansion of granulocytic MDSCs in the colon, high levels of arginase 1 and increased production of anti-inflammatory Th2 cell cytokines (83). Thus, the study of STAT3-CEBPβ signaling pathway in BM-MDSCs *per se* could elucidate the underlying mechanisms in colitis pathogenesis and open the avenue for using them as promising cell-based therapeutic option for IBD patients.

Taken together, our results indicate that *in vitro* generated BM-MDSCs bear suppressive capacity *in vitro* and *in vivo* under steady state, but fail to control colitis *in vivo*. Their intrinsic plasticity renders them prone to *in vivo* conversion into effector cells and consequent loss of their suppressive potential under inflammatory conditions. To allow application of MDSCs as immunotherapy, we could envision them as maintenance therapy to prevent disease reactivation following induction of remission with other therapeutic factors.

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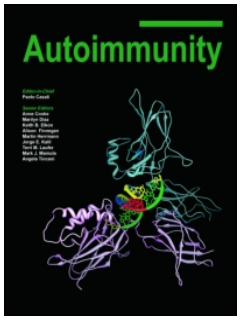
10. ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to my project supervisor, Panos Verginis for his guidance. Without his support it would not be possible to conduct this study.
Besides my advisor, I would like to thank Prof. Dimitrios Boumpas who provided me the opportunity to join his team and gave access to the laboratory and research facilities.
I also want to thank Prof. Kontastinos Papadakis who helped me recruit patients and collect data for this thesis.
A special thank to Prof. Ioannis Mouzas for serving as chairman of my committee even at hardship. Your support has been priceless.
I would like to thank my labmates and friends, especially Marianna, Ilias, Eirini and Christianna not only for working together but also for all the fun we had.
I would further like to acknowledge Xaroula and Niki, the sorting facility at IMBB for their assistance and support throughout my study.
Special thanks to my friends DIMITRA T, Dimitra V, Ilias, Ioanna, Nikos, Eleni, Maro, George S, George P, Nikos and Alexandros who made my stay at Heraklion magnificent.
Last but not least, I would like to thank my birth family, and my friends (Dimitra T and Dimitris V) that I call family for everything they do for me, for what each of them stands for.



Autoimmunity

ISSN: 0891-6934 (Print) 1607-842X (Online) Journal homepage: http://www.tandfonline.com/loi/iaut20

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To cite this article: Eleni Kontaki, Dimitrios T. Boumpas, Maria Tzardi, Ioannis A. Mouzas, Konstantinos A. Papadakis & Panayotis Verginis (2017): Aberrant function of myeloid-derived suppressor cells (MDSCs) in experimental colitis and in inflammatory bowel disease (IBD) immune responses, Autoimmunity

To link to this article: http://dx.doi.org/10.1080/08916934.2017.1283405

Published online: 03 Mar 2017.

Autoimmunity

http://informahealthcare.com/aut
ISSN: 0891-6934 (print), 1607-842X (electronic)

Autoimmunity, Early Online: 1–12
 DOI: 10.1080/08916934.2017.1283405

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ORIGINAL ARTICLE

Aberrant function of myeloid-derived suppressor cells (MDSCs) in experimental colitis and in inflammatory bowel disease (IBD) immune responses

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Abstract

Background and aims: Myeloid-derived suppressor cells (MDSCs) encompass a novel population of suppressor cells and a potential candidate for cell-based therapies in inflammatory diseases. Herein, we investigated their immunomodulatory properties in experimental inflammatory colitis and T cell-mediated immune responses in inflammatory bowel disease (IBD) patients. *Methods*: MDSCs (defined as $CD14^{-}HLA^{-}DR^{-/low}CD33^{+}CD15^{+}$) numbers were determined in peripheral blood (PB) from IBD patients. PB MDSC function was assessed *in vitro*. Experimental colitis was induced upon 2,4,6-trinitrobenzene sulfonic acid (TNBS) treatment and MDSCs were characterized by flow cytometry. The *in vivo* suppressive potential of bone marrow (BM)-derived MDSCs (BM-MDSCs) was tested by using both depleting and adoptive transfer strategies. *Results*: MDSCs were enriched in the periphery of IBD patients during active disease. TNBS colitis induced amplification of MDSCs, particularly of the granulocytic ($Ly6G^{+}$) subset during the effector phase of disease. Of interest, BM-MDSCs potently suppressed $CD4^{+}$T cell responses under steady state but failed to control colitis-associated immune responses *in vivo*. Mechanistically, under the colonic inflammatory milieu MDSCs switched phenotype (decreased proportion of $Gr1^{high}$ and increased numbers of $Gr1^{low}$) and downregulated CCAAT/enhancer-binding protein beta (CEBPβ) expression, a critical transcription factor for the suppressive function of MDSCs. In accordance with the murine data, human $CD33^{+}CD15^{+}$MDSCs from peripheral blood of IBD patients not only failed to suppress autologous T cell responses but instead enhanced T cell proliferation *in vitro*. *Conclusions*: Our findings demonstrate an aberrant function of MDSCs in experimental inflammatory colitis and in IBD-associated immune responses *in vitro*. Delineation of the mechanisms that underlie the loss of MDSCs function in IBD may provide novel therapeutic targets.

Keywords

MDSCs, BM-MDSCs, IBD, experimental colitis, CEBPβ

History

Received 7 July 2016
Revised 4 December 2016
Accepted 7 January 2017
Published online 2 March 2017

Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing disease that results from an aberrant immune response to intraluminal bacterial antigens in genetically predisposed subjects [1–3]. CD is widely considered to result from exaggerated Th1 and Th17 immune responses, as well as decreased Treg responses [4–7]. However, the mechanisms underlying the dysfunctional T cell responses remain largely unknown. Myeloid derived-suppressor cells (MDSCs) represent a key cell population regulating immune responses [8]. MDSCs are a diverse population comprised of myeloid precursors of granulocytes, macrophages, and dendritic cells. In mice, MDSCs are characterized by the co-expression of Gr-1 and CD11b markers, and are further divided into granulocytic and monocytic subset, defined as $CD11b^{+}Ly6G^{+}Ly6C^{low}$ and $CD11b^{+}Ly6G^{-}Ly6C^{+}$cells, respectively [8–11]. In humans, phenotypic characterization of MDSCs is challenging, due to the lack of uniform criteria. Nevertheless, they are most commonly characterized by the expression of myeloid marker CD33 and the lack of expression of both the major histocompatibility complex (MHC) class-II molecule HLA-DR and several other lineage markers [12–15]. Although most of our knowledge on the role of MDSCs in immune responses has been based on studies with tumor bearing mice and cancer patients [8,16–19], increasing evidence has suggested their role in many pathological conditions, such as infections [16,20],

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transplantation [21–23], and autoimmunity [14,15,24–28]. Traditionally, MDSCs are considered as potent T cell suppressors, through cell–cell contact and release of soluble mediators [29,30]. However, recent literature highlighted their plasticity, denoting the proinflammatory potential of specific subsets emerging in specific microenvironment [31].

Despite the well-defined suppressive effects of MDSCs on T cell responses, their function in autoimmune diseases, like IBD, is controversial. In particular, the immunosuppressive function of MDSCs was suggested by several reports showing that $CD11b^+Gr1^+$ MDSCs are increased during intestinal inflammation [32–34]. As is the case for murine colitis models, $CD14^+HLD\text{-}DR^{low}$ MDSCs with suppressive properties were also reported to be increased in the peripheral blood of IBD patients [33]. On the other hand, recent studies supported a proinflammatory role of myeloid cells in experimental colitis, demonstrated that adoptively transferred BM $Ly6C^{high}$ cells differentiated into inflammatory cells, contributing to intestinal inflammation [35–37]. In this study, we sought to dissect the contribution of MDSCs in the regulation of IBD and explore their therapeutic efficacy and its mechanism. Herein, we demonstrate a significant expansion of granulocytic MDSCs during active TNBS colitis. However, adoptive transfer of BM-MDSCs fails to suppress immune response *in vivo*. Interestingly, the colitic environment favors MDSCs phenotype switch and downregulation of CEBPβ expression, a master regulator of MDSCs suppressive function. Importantly, human MDSCs from peripheral blood of patients with IBD are also aberrant enhancing autologous T cell proliferation. Together, our data provide evidence that colitic milieu favors the conversion of MDSCs from suppressor to inducer cells in both experimental and human colon inflammation acquiring a proinflammatory profile.

Materials and methods

IBD patients

IBD patients were recruited through the Gastroenterology Department, University Hospital of Heraklion (Crete, Greece). The disease's diagnosis and classification was established by clinical criteria and endoscopic/histopathological findings of World Gastroenterology Organization Global Guidelines. Disease activity was determined by Ulcerative Colitis Activity Index and Harvey–Bradshaw Crohn's disease activity index. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete) approved this study.

Mice

Female Balb/c mice (8–10 week) were obtained from the specific pathogen-free facility of the Institute of Molecular Biology and Biotechnology (Heraklion, Crete, Greece). DO11.10 TCR transgenic mice were kindly provided by Dr. Panoutsakopoulou (Biomedical Research Foundation of the Academy of Athens, Athens, Greece). All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office.

Reagents

For human cell phenotypes, the following fluorescent-conjugated mAbs were used: CD15 (8OH5), CD33 (D3HL60.251), CD14 (RM052), and CD25 (B1.49.9) from Beckman Coulter, Fullerton, CA, HLA-DR (L243, G46-6) and CD4 (RPA-T4) from BD Pharmigen, San Diego, CA. For analysis of mouse cells the following mAbs were used: Ly6C (1G7.G10) from Miltenyi Biotec, Bergisch Gladbach, Germany, Gr-1 (RB6- 8C5) from eBioscience, San Diego, CA, CD11c (N418) from BioLegend, San Diego, CA, CD11b (M1/70), CD3e (145-2C11), CD19 (1D3), CD4 (RM4-5), Ly6G (1A8), CD44 (Pgp-1, Ly24), and CD25 (PC61) from BD Pharmigen, San Diego, CA.

Cell cultures were performed in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640, both supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 2-mercaptoethanol (2-ME) (5×10^{-5} M) all from Gibco, Carlsbad, CA. TNBS was purchased from Sigma-Aldrich, St. Louis, MO.

Human mononuclear cell isolation from peripheral blood

Heparinized blood was collected from healthy subjects and IBD patients and peripheral blood mononuclear cells (PBMCs) were isolated on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) density gradient. MDSCs and $CD4^+$T cells were analyzed by flow cytometry and sorted as described. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete) approved this study.

Colitis induction, *in vivo* depletion of MDSCs and adoptive-transfer experiments

Acute TNBS acute colitis was induced by intrarectal injection of 2–3 mg TNBS dissolved in 40% ethanol, as described previously [38]. Mice were monitored daily for weight loss. Five days after TNBS delivery mice were killed. Spleens and mesenteric lymph nodes (MLNs) were removed and processed appropriately.

Depletion of MDSCs was achieved by injecting intraperitoneally (i.p.) 250 μg anti-Gr1 mAb (RB6-8C5) (BioLegend, San Diego, CA) on days 0 and 2 (day 0: TNBS instillation). Efficient depletion was confirmed by FACS analysis on homogenized spleen suspension on day 5.

In the adoptive-transfer experiment, either *in vitro* generated from naïve BM or *ex vivo* isolated from BM of murine colitis live 7-aminoactinomycin D $(7AAD)^-CD3^-CD19^-CD11b^+Gr1^+$ cells were sorted (purity $>90\%$) and 2×10^6 cells were transferred intravenously (i.v.) into TNBS-instilled mice on days 0 and 2 after TNBS administration. Mice were monitored daily for clinical signs of disease.

Lamina propria mononuclear cell (LPMC) isolation

LPMCs were isolated as described previously [39]. After de-epithelization, colonic tissue was resuspended in Hanks' Balanced Salt Soln (HBSS) with collagenase D 0.5 mg/ml and DNase I 0.1 mg/ml (Sigma-Aldrich, St. Louis, MO) for 60 min

at 37 °C. LPMCs were purified on a 30%/70% discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, MO).

Flow cytometry and cell sorting

Single cell suspensions were prepared from tissues and cells were stained for extracellular markers for 20 min at 4 °C in 5% Fetal Calf Serum (FCS) (Gibco, Carlsbad, CA) in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO). Dead cells were identified and excluded from all analyses by 7AAD (BD Pharmigen, San Diego, CA). Events were acquired on a FACS-Calibur (BD Biosciences, San Diego, CA) and analyzed using the FlowJo software (Tree Star, San Carlos, CA). Cell sorting was performed using the high–speed MoFlo cell sorter (Dako, Santa Clara, CA).

Generation of $CD11c^{-}CD11b^{+}Gr1^{+}$ cells from bone marrow *in vitro* and cytokine assessment

MDSCs were generated as previously described [40]. Tibias and femurs from naïve Balb/c mice were removed and BM was flushed out. Red blood cells (RBCs) were lysed with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). To obtain BM-derived MDSCs, 2.5×10^6 cells were plated into 100 mm dishes in 10 mL of complete medium supplemented with 30% supernatant from a murine granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting X63Ag8 cell line (kindly provided by Dr. Eliopoulos AG, Molecular and Cellular Biology Laboratory, University of Crete Medical School, Heraklion, Greece). Cells were maintained at 37 °C in 5% CO_2-humidified atmosphere. After 3 days, non-adherent cells were collected and $CD11c^{-}CD11b^{+}Gr1^{+}$ cells were purified by sorting. IL-10 was measured in supernatants of cell cultures by ELISA (BD Opt EIA; BD Biosciences, San Diego, CA).

In vitro suppression assays

Naïve MLNs cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (eBioscience, San Diego, CA) (1 μM for 10 min at 37 °C in labeling buffer 0.1% Bovine Serum Albumin-BSA in PBS) (Sigma-Aldrich, St. Louis, MO) and cocultured (2×10^4 cells/well) with sorted BM-MDSCs (purity > 95%), at 1:1 ratio, in the presence of 2 μg/ml plate bound anti-CD3 (145-2C11; BD Biosciences, San Diego, CA) and 1 μg/ml anti-CD28 (37.51; BD Biosciences, San Diego, CA). Activation of $CD4^{+}$T cells was determined based on CD25 expression, while proliferation based on CFSE dilution by flow cytometry. Similarly, human purified CFSE-labeled $CD4^{+}CD25^{-}$ T cells from PBMCs, were cocultured with autologous sorted MDSCs from IBD patients at 1:1 ratio, in the presence of plate bound anti-CD3 (OKT) and anti-CD28 (CD28.2), both purchased from eBioscience, San Diego, CA. Proliferation of T cells was determined based on CFSE dilution by flow cytometry.

In vivo suppression assay

Under the combined anesthesia with ketamine (Merial, Milano, Italy) and xylazine (Beyer, Leverkusen, Germany), spleens of naïve Balb/c mice were injected with and exposed to T cells mixed with equal numbers of antigen presenting cells (APCs), in the presence or absence of BM-MDSCs (at 1:1 ratio). $CD4^{+}$T cells enriched from spleens of DO11.10 Tg mice by sorting were labeled with CFSE. Sorted dendritic cells (DCs) isolated from spleen of naïve Balb/c mice were primed *in vitro* with lipopolysaccharide (LPS) (0.25 μg/ml; Invivogen, San Diego, CA) and loaded with the ovalbumin (OVA) antigenic peptide (amino acids 323–339) at 20 μg/ml (Sigma-Aldrich, St. Louis, MO) for 2 h at 37 °C. $Gr1^{+}$ $CD11b^{+}$BM-MDSCs were generated *in vitro* as described previously. On day 3, activation and proliferation of the transferred $CD4^{+}$T cell population were assessed based on CD44 expression and CFSE dilution, respectively, by flow cytometry.

Histology

Mouse colons were dissected out, a piece of the proximal and one from the distal colon were fixed in 10% neutral buffered formalin, and embedded in paraffin. Transverse sections (6–7 μm) from colons were stained with hematoxylin and eosin (H&E), for histological analysis using a Nikon Eclipse E800 microscope (Tokyo, Japan).

Western blot for CEBPβ detection

Whole-cell lysates (40 μg protein) were subjected to SDS-PAGE electrophoresis on 10% gels and then transferred to nitrocellulose membranes (Protran; Whatman, GE Healthcare, Chalfont St. Giles, UK). Membranes were blocked with 5% milk in Tris-Buffered Saline Tween-20 (TBST) and then incubated with anti-CEBPβ Ab (1:1000; Abcam, Cambridge, UK), as well as anti-actin (1:2000; Sigma-Aldrich, St. Louis, MO) as a loading control. Detection was performed by using HRP-conjugated anti-Ig (Sigma-Aldrich, St. Louis, MO) and chemiluminescent reagents (Supersignal Substrate; Pierce, Waltham, MA).

Statistics

Two-tailed Student's *t* tests were carried out and p values are reported. Mann–Whitney *U* test was used for the statistical analysis of human MDSCs. Kaplan–Meier survival analysis was used to examine the impact of cell transplantation on the survival of the mice. All analyzes were performed using the GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

$CD33^{+}CD15^{+}$ MDSCs are enriched in IBD patients

We initially screened IBD patients for the presence of MDSCs. Human MDSCs have mainly been described in patients with cancer and are phenotyped as $HLA\text{-}DR^{-/low}$ $CD14^{-}CD33^{+}CD15^{+}$ [12–15]. We stratified CD and UC patients into active and quiescent cohort and compared MDSCs of these groups to that of healthy controls (demographics in Table 1). Flow cytometry analysis demonstrated significantly increased frequency (Figure 1A) and relative numbers (Figure 1B) of $HLA\text{-}DR^{-/low}CD14^{-}CD33^{+}$ $CD15^{+}$cells in both patients with active CD ($p = 0.0008$) and UC ($p = 0.009$) compared with healthy controls. In contrast, no elevation of MDSCs in patients with quiescent CD ($p = 0.1$) or UC ($p = 0.2$) was observed compared to the

Table 1. Patients characteristics.

Type of disease	Stage of disease	No.	Male	Female	Average age (year)
Ulcerative Colitis	Active	12	19	6	47.9
	Remission	13			
Crohn's Disease	Active	11	14	8	45.3
	Remission	11			
Healthy		19	10	9	34.3

levels in healthy controls. However, within the CD ($p = 0.1$) and UC ($p = 0.08$) groups, no statistically significant difference was detected with respect to MDSCs levels (Figure 1B).

MDSCs expand during TNBS colitis course

To delineate the role of MDSCs in intestinal inflammation, we used the TNBS mouse model of colitis, a commonly used animal model that resembles human Crohn's disease [38]. Mice receiving TNBS developed severe colitis with an active

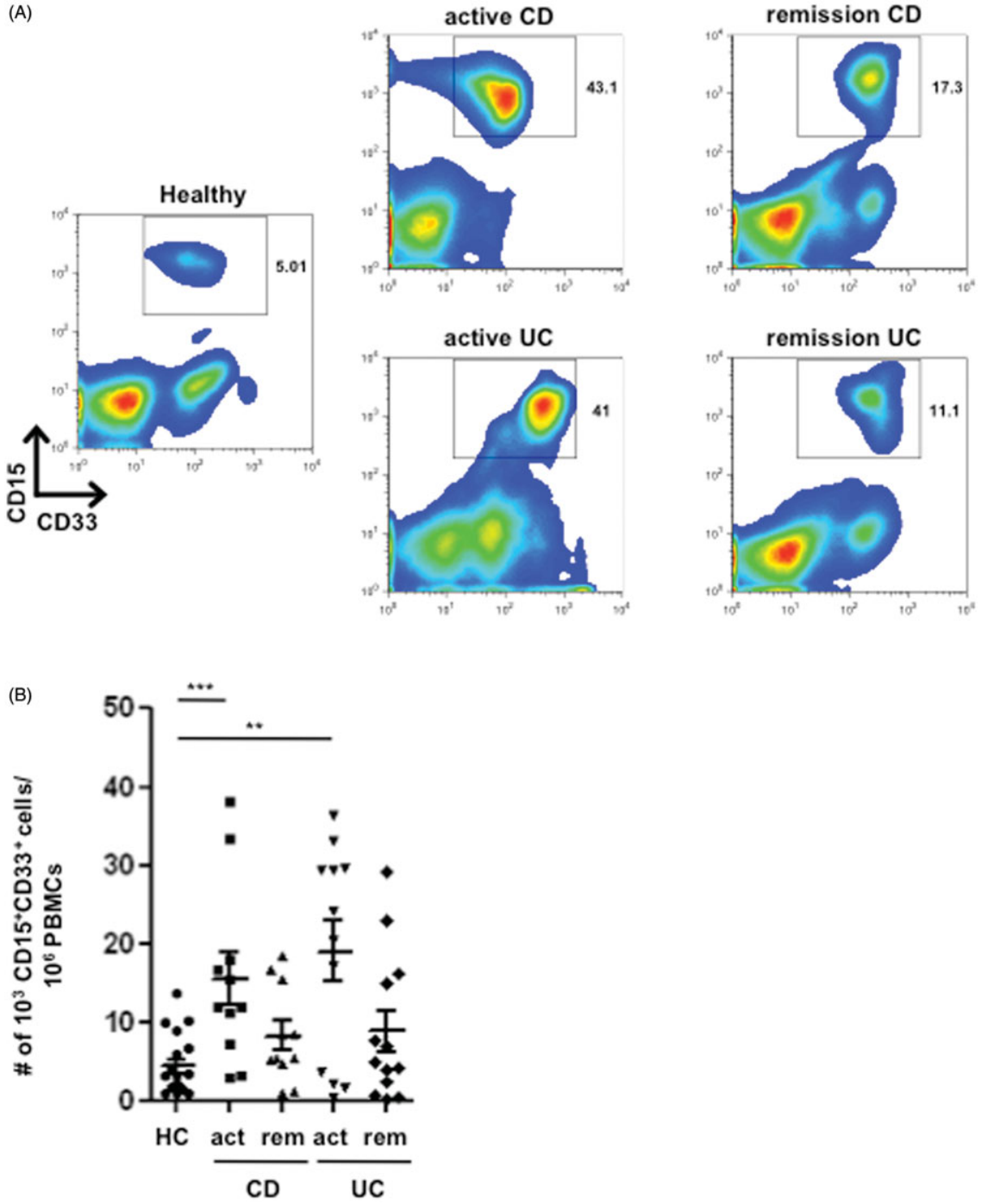

Figure 1. MDSCs are enriched in IBD patients. Frequency (A) and relative numbers (B) of $CD33^+CD15^+$ MDSCs in the peripheral blood of CD ($n = 22$) and UC ($n = 25$) patients with active disease or in remission, as well as healthy controls ($n = 19$). Gates were set on HLA-$DR^{-/low}$ $CD14^-CD33^+CD15^+$ cells. Mann–Whitney U test p values: **, $p < 0.01$; ***, $p < 0.001$. HC, healthy controls; act, active; rem, remission.

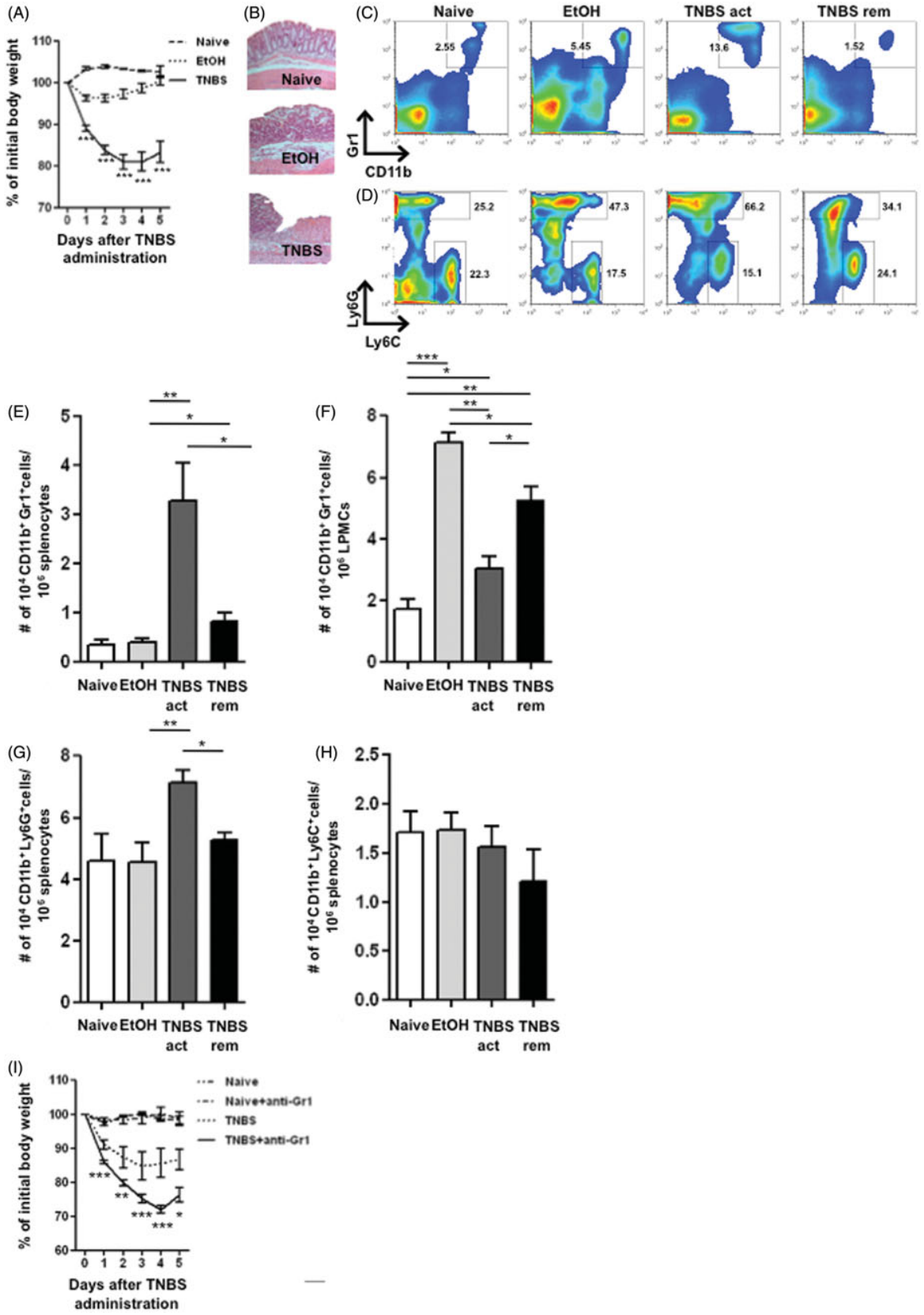

Figure 2. The role of MDSCs in TNBS-induced colitis. Mice were intrarectally administered 2–3 mg TNBS per mouse on day 0. (A) Body weight changes after TNBS instillation, and (B) H&E staining of colonic sections (×10). $CD11b^+Gr1^+$ MDSCs (C) and Ly6G/C-MDSCs (D) accumulation in the spleen of mice during TNBS colitis. Representative flow cytometric analysis indicates percentages of MDSCs. Gates were set on $7AAD^-CD19^-CD3^-$ or $7AAD^-CD19^-CD3^-CD11b^+$ cells for C and D, respectively. Relative numbers of $CD11b^+Gr1^+$ MDSCs/10^6 splenocytes (E) and $CD11b^+Gr1^+$ MDSCs/10^6 LPMCs (F), $Ly6G^+$ (G), and $Ly6C^+$ (H) MDSCs/10^6 splenocytes during the different phases of the disease. Data are shown as mean ± SD ($n = 3$–6 mice/group). (I) Mice received 250 µg of anti-Gr1 mAb (RB6-8C5) i.p. on days 0 and 2. TNBS colitis was induced on day 0. Body weight of mice subjected to TNBS colitis treated with anti-Gr1 mAb. Data shown represent pooled values from four independent experiments. *T* test *p* values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. act, active; rem, remission.

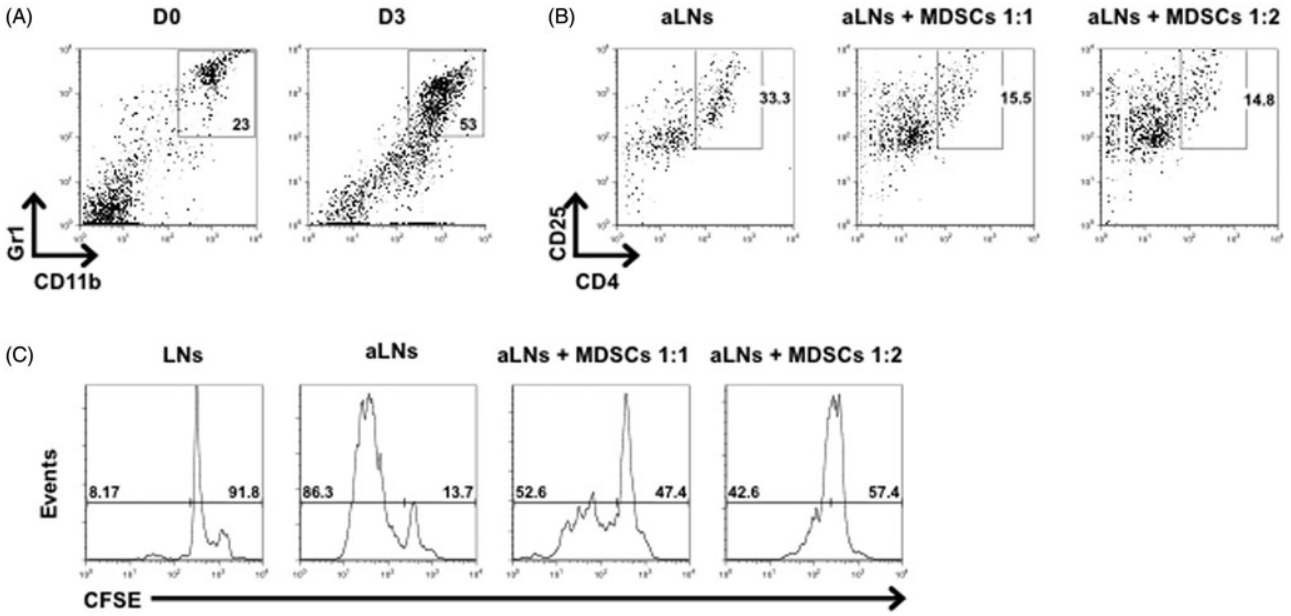

Figure 3. *In vitro* enrichment of BM-MDSCs. Culture of BM cells with high doses of GM-CSF for 3 d evoked efficient enrichment of the $Gr1^+CD11b^+$ population *in vitro* (A). Sorted $Gr1^+CD11b^+$ BM-MDSCs were cocultured with CFSE-labeled MLN cells, activated by plate bound anti-CD3 and soluble anti-CD28. $CD4^+$ T cell activation and proliferation was measured by flow cytometry on day 5. Dot plots show CD4 versus CD25 on gated viable $CD4^+$ T cells (B). CFSE dilution of $CD4^+$ T cells. Numbers indicate the percentages of cells that proliferated (C). Results are representative of three independent experiments.

phase characterized by bloody diarrhea and extensive weight loss until day 5, and followed by gradual weight recovery reflecting inflammation resolution (Figure 2A). Histological analysis showed that TNBS colitis was characterized by loss of architecture and marked inflammatory infiltration (Figure 2B). Using this mouse model, we assessed the expansion of $Gr1^+CD11b^+$ MDSCs in the spleen during the course of TNBS colitis. The frequency (Figure 2C), as well as the relative numbers (Figure 2E) of $Gr1^+CD11b^+$ MDSCs ($7AAD^-CD19^-CD3^-$) was significantly increased during the effector phase of colitis, while they contracted when mice entered remission. Interestingly, the relative numbers of $Gr1^+CD11b^+$ MDSCs were increased in colon LPMCs, during inflammation resolution (Figure 2F). In parallel, $CD11b^+$ cells present in the spleen of TNBS-treated mice were further analyzed for expression levels of Ly6G and Ly6C. Between the two subsets of MDSCs, only $Ly6G^+$ ($7AAD^-CD19^-CD3^-CD11b^+$) MDSCs resembled the expansion of $Gr1^+CD11b^+$ MDSCs during colitis course that is they were increased during the active phase and declined during inflammation resolution. In contrast, $Ly6C^+$ MDSCs were not significantly altered throughout the disease progress (Figure 2D, G, and H). These data demonstrated that TNBS-induced colitis is characterized by a rapid, but transient increase of MDSCs in the spleen, suggesting a potential role of these cells in the regulation of immune response.

To further investigate the contribution of MDSCs to TNBS-induced colitis, we depleted MDSCs through administration of the anti-Gr1 mAb to TNBS-treated mice on days 0 and 2. Although, anti-Gr1 mAb is not selective for depletion of MDSCs, since it could also deplete neutrophils, this is a commonly used strategy eliminating MDSCs in various murine models of cancer and autoimmunity [41–43]. Interestingly, Gr1 depletion from the periphery of TNBS-treated mice rather aggravated the colitic phenotype (Figure 2I). Similarly, Gr1 depletion after colitis establishment (administration of anti-Gr1 mAb on days 3 and 5) resulted in a more severe phenotype compared to TNBS-treated mice (data not shown).

BM-MDSCs exert suppressive function under steady state

To understand whether the colitis milieu affects MDSCs function, we utilized a protocol for *in vitro* generation of BM-MDSCs, as described by Rossner et al. [40]. To this end, MDSCs were harvested as non-adherent cells from BM cell cultures supplemented with GM-CSF. Indeed, culture of BM cells with high doses of GM-CSF evoked efficient enrichment of the $Gr1^+CD11b^+$ population *in vitro* as depicted in Figure 3(A). Although enriched *in vitro*, it remained to be determined whether BM derived $Gr1^+CD11b^+$ cells, could exert suppressive function. To address this, sorted BM-$Gr1^+CD11b^+$ cells – enriched by GM-CSF – were co-cultured with autologous, CFSE-labeled MLN cells stimulated with anti-CD3/CD28. After 5 d of coculture, BM-$Gr1^+CD11b^+$ cells potently suppressed activation (Figure 3B) and proliferation of responder $CD4^+CD25^-$ T cells in a dose dependent manner (Figure 3C).

To investigate whether BM-MDSCs could suppress immune responses *in vivo*, naïve Balb/c mice were intrasplenically injected with CFSE-labeled DO11.10 T cells, ovalbumin-pulsed splenic dendritic cells, denoted as DC.OVA, and/or $CD11b^+Gr1^+$ MDSCs harvested from BM. At 72 h, activation and proliferation of $CD4^+$ T cells were analyzed using flow cytometry. Mice treated with DC.OVA plus BM-MDSCs exerted decreased proliferation and activation (Figure 4A,

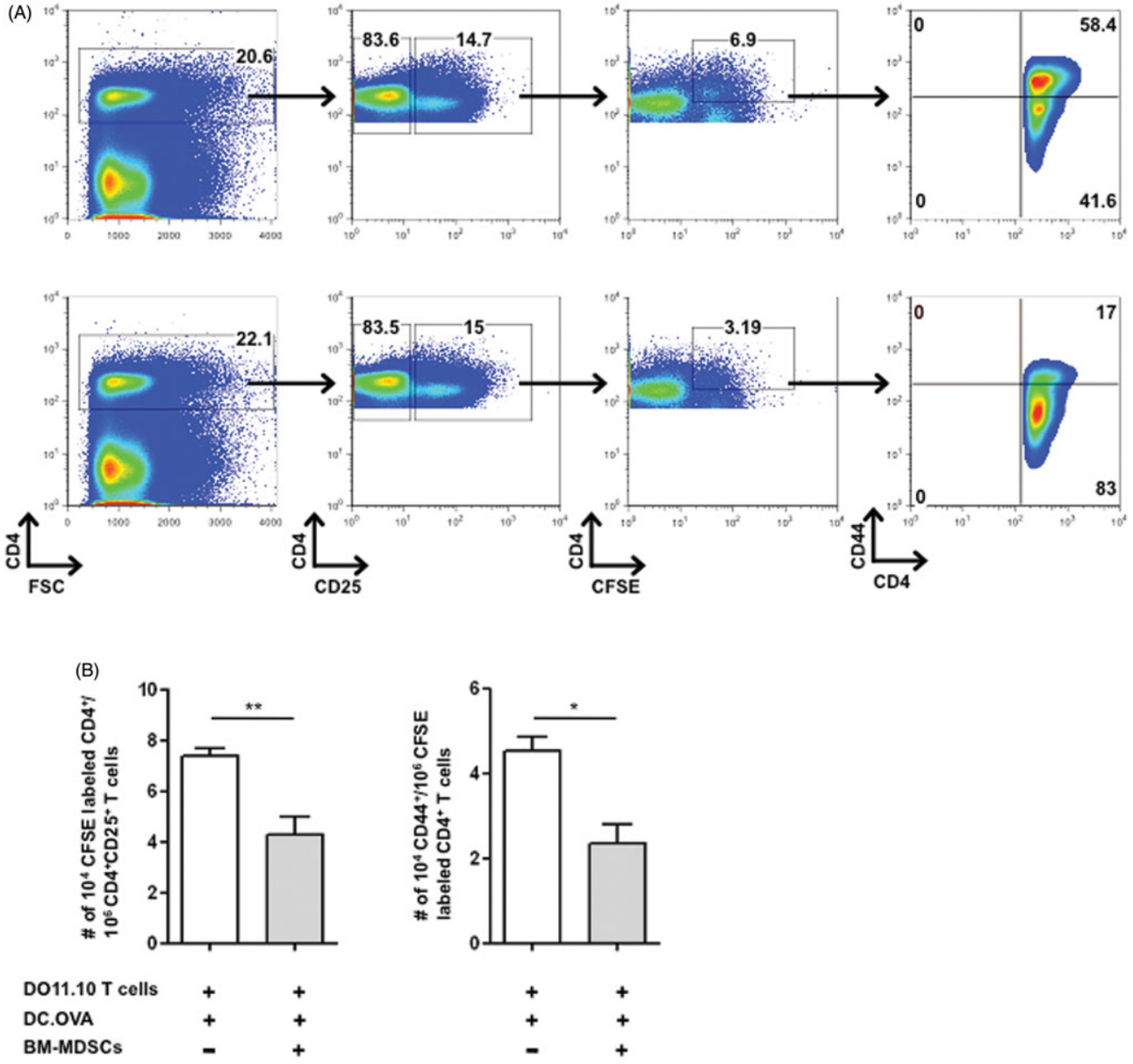

Figure 4. BM-MDSCs suppress T cell responses under steady state *in vivo*. (A) CFSE-labeled DO11.10 T cells (5×10^4) and DC.OVA (5×10^4) were intrasplenically injected into naïve Balb/c mice alone (upper panel) or with BM-MDSCs (5×10^4) (lower panel). On day 3, flow cytometry was performed. $CD4^+CD25^+$ T cells were gated and the fraction of CFSE-labeled $CD4^+$ T cells was determined. In the same experiment, CFSE-labeled $CD4^+$ T cells were analyzed for the expression of CD44. Experiments were performed with two mice/groups. (B) Relative numbers of CFSE-labeled $CD4^+$ T cells/10^6 $CD4^+CD25^+$ T cells (left panel) and $CD44^+$ T cells/10^6 CFSE-labeled $CD4^+$ T cells (right panel). Data are shown as mean ± SD ($n = 3$–5 mice/group). *T* test *p* values: *, $p < 0.05$; **, $p < 0.01$.

lower panel) compared to mice treated with DC.OVA alone (Figure 4A, upper panel), as indicated by CFSE dilution and CD44 – another T cell activation marker instead of CD25 – expression, respectively. MDSC-mediated suppression of T cell proliferation and activation was significant, as extrapolated by enumeration of the $CD4^+$ T cells (Figure 4B). Collectively, these data suggest that under steady-state BM-MDSCs suppress T cell activation and proliferation both *in vitro* and *in vivo*.

BM-MDSCs fail to suppress ongoing colitis *in vivo*

To determine whether BM-MDSCs sustain their suppressive function *in vivo* under inflammatory conditions, highly pure BM-derived $Gr1^+CD11b^+$ MDSCs were adoptively transferred in TNBS-treated mice. Surprisingly, adoptive transfer of two doses of BM-MDSCs did not protect against colitis, but rather augmented the colitic phenotype, as indicated by the increased mortality rate (Figure 5A) and weight loss (Figure 5B) compared with TNBS only-treated mice. Furthermore, adoptive transfer of MDSCs isolated from the BM of TNBS-instilled mice into syngeneic hosts with ongoing colitis, failed to regulate disease (Figure 5C). These data suggest that the inflammatory environment of TNBS colitis switched off the suppressive capacity of BM-MDSCs.

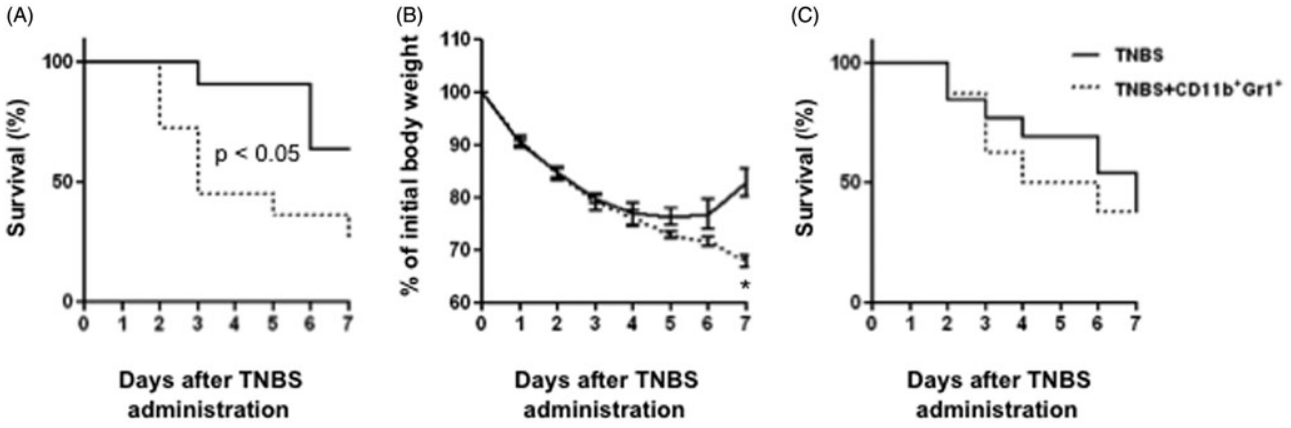

Figure 5. BM-MDSCs transfer aggravates TNBS colitis. TNBS-treated mice were i.v. injected with sorted *in vitro* generated $Gr1^+CD11b^+$ BM-MDSCs (2×10^6/mouse, purity > 90%) on days 0 and 2 after TNBS administration. Mortality rate shown as Kaplan–Meier analysis (A) and body weight loss (B) of $Gr1^+CD11b^+$ BM-MDSCs treated and control mice. Data are shown as mean ± SD ($n = 11$ mice/group). TNBS-treated mice were i.v. injected with sorted *ex vivo* derived $Gr1^+CD11b^+$ cells (2×10^6/mouse, purity > 90%) isolated from BM of TNBS-instilled mice with ongoing disease on days 0 and 2 after TNBS administration. Mortality rate shown as Kaplan–Meier analysis (C) of $Gr1^+CD11b^+$ treated and control mice. Combined results of two independent experiments are shown ($n = 8$–13 mice/group). *T* test *p* values: *, $p < 0.05$.

Characterization of BM-MDSCs in colitic environment

To explore the mechanism by which BM-MDSCs lost their suppressive properties in the TNBS colitis milieu, fresh BM cells, enriched by GM-CSF, were cultured in the presence of serum from either naive or TNBS-treated mice. On day 3, cells were analyzed by flow cytometry. Importantly, serum from TNBS-treated mice led to a decreased proportion of $CD11b^+Gr1^{high}$, accompanied by increased relative numbers of $CD11b^+Gr1^{inter}$ cells (Figure 6A). Looking on $Ly6G^+$ no such differences were observed (Figure 6B). Furthermore, BM-MDSCs exposed to TNBS serum produced significantly lower levels of IL-10, a key anti-inflammatory cytokine, compared with untreated BM-MDSCs (Figure 6C).

Recent data suggest that CEBPβ controls the immunosuppressive function of MDSCs, linking the absence of CEBPβ transcriptional regulation to the loss of suppressive activity [22]. In accordance with this study, CEBPβ was barely detectable in freshly isolated $Gr1^+CD11b^+$, $CD11b^+Ly6G^{high}$ or $CD11b^+Ly6G^{low}$ BM cells. Interestingly, with the exception of $CD11b^+Ly6G^{high}$ cells, neither of the subsets of *in vitro* generated BM-MDSCs expressed CEBPβ. Even in the case of MDSCs isolated from BM of TNBS-treated mice, CEBPβ could not be detected, further supporting the idea that BM-MDSCs lose their suppressive properties under the specific inflammatory environment (Figure 6D).

Human MDSCs promote T cell proliferation in IBD patients

We next evaluated the suppressive ability of $CD33^+CD15^+$ MDSCs from patients with IBD. To this end, purified MDSCs ($CD33^+CD15^+HLA\text{-}DR^{-/low}$ $CD14^-$) (Figure 7B) from peripheral blood of IBD patients were added to autologous sorted CFSE-labeled $CD4^+CD25^-$ T cells (Figure 7A), stimulated with anti-CD3/CD28 and proliferation was analyzed on day 5. Surprisingly, $CD33^+CD15^+$ MDSCs not only failed to suppress, but rather promoted T cell proliferation, as indicated by CFSE dilution (Figure 7C). Notably, the percentage (Figure 7D) and total numbers (Figure 7E) of undivided $CD4^+$ T cells is diminished upon addition of MDSCs in the culture, suggesting that MDSCs markedly promoted the proliferation of T cells. Collectively, these results highlight the important role of MDSCs in IBD course, but also raise an intriguing question concerning their function.

Discussion

Restoration of immune homeostasis and self-tolerance represent the ultimate goal in IBD therapy. A comprehensive understanding of the interactions between several immune cells contributing to the intestinal inflammation should provide insights into the design of immunotherapeutic interventions. In this study, we show that the frequency of MDSCs increased dramatically in the periphery of IBD patients upon exacerbation of disease. Interestingly, although amplified, these cells failed to suppress autologous T cell proliferation *in vitro*. Accordingly, mouse studies demonstrate an expansion of MDSCs, particularly the granulocytic subset, in the peripheral lymphoid compartment, during active colitis. Importantly, *ex vivo* expanded BM-MDSCs, which potently suppress T cell responses under steady state, failed to abrogate the colitic phenotype, when adoptively transferred to TNBS-treated mice. Under such an inflammatory environment, BM-MDSCs switched phenotype and downregulated CEBPβ expression, a master regulator of the immunosuppressive function of MDSCs. Together, our results indicate that although active colitis induces recruitment of MDSCs, they are not competent to effectively limit autoimmune T cell responses.

It is rather difficult to demonstrate whether MDSCs described in this article represent a subset of neutrophils that have regulatory functions under pathologic conditions or a distinct myeloid cell population. Although granulocytic MDSCs and ''regular'' neutrophils share similar phenotypic traits, it has been proposed that they also possess important differences. For example, granulocytic MDSCs could be distinguished from neutrophils based on the morphology of the nucleus, with the former having ring-shaped appearance

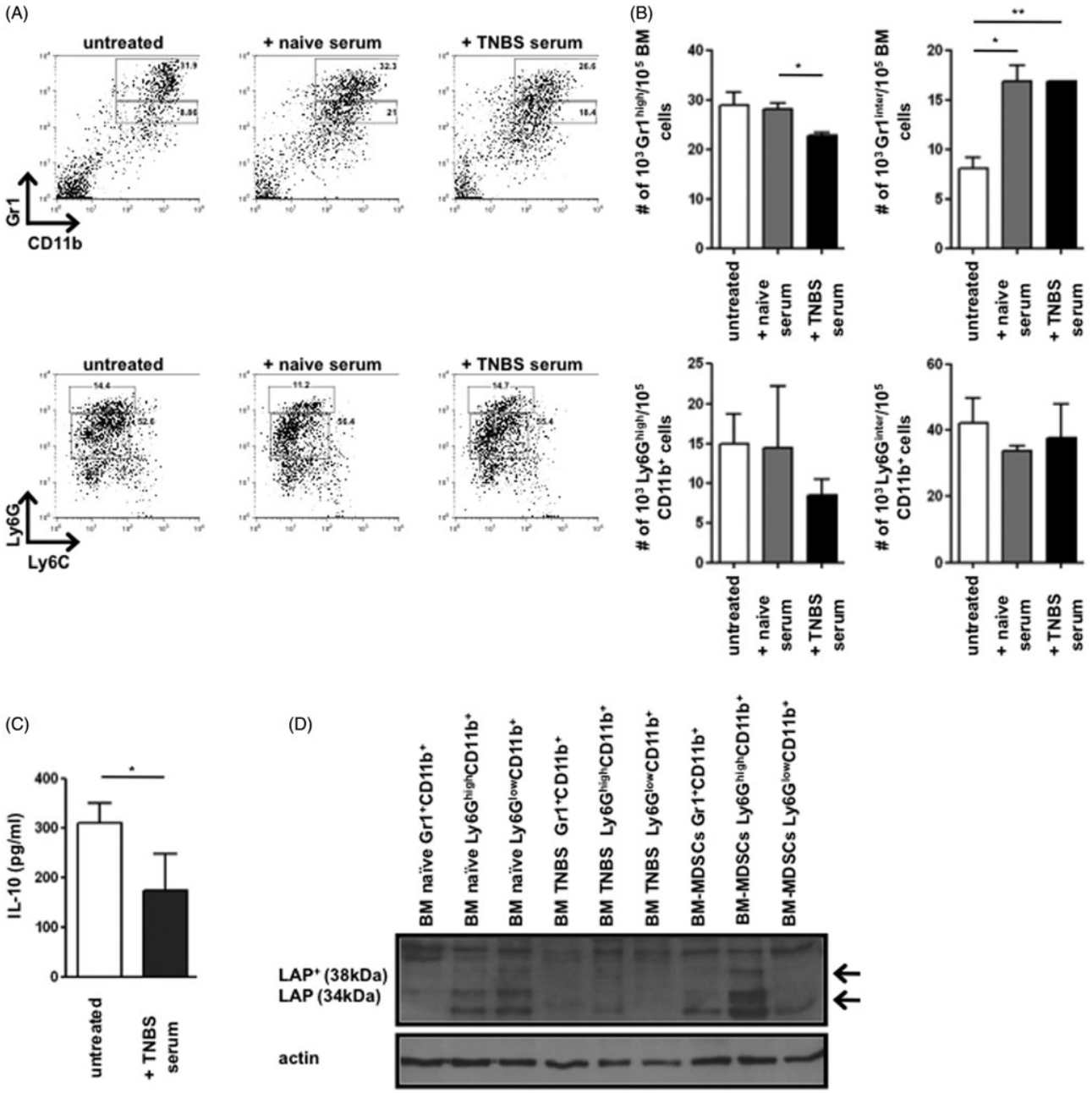

Figure 6. Phenotypic characterization of BM-MDSCs in colitic environment. Freshly isolated BM cells were cultured with GM-CSF in the presence of serum harvested either from naïve or active colitic mice. Untreated BM-MDSCs were used as controls. On day 3, frequency and relative numbers (mean ± SD) of $Gr1^{high}CD11b^{+}$, $Gr1^{low}CD11b^{+}$ (A), $Ly6G^{high}$, $Ly6G^{low}$ BM-MDSCs (B) were assessed by using flow cytometry. Gates were set on $7AAD^{-}CD19^{-}CD3^{-}CD11c^{-}$ and $7AAD^{-}CD19^{-}CD3^{-}CD11b^{+}$ cells. (C) Supernatants were assessed for IL-10 production by ELISA. Data are representative of three independent experiments. *T* test *p* values: *, $p < 0.05$; **, $p < 0.01$. (D) CEBPβ expression was assessed by western blot analysis in sorted BM-derived $Gr1^{+}CD11b^{+}$, $Ly6G^{high}$, and $Ly6G^{low}$ cells either from naïve or TNBS-treated mice and *in vitro* generated BM $Gr1^{+}CD11b^{+}$, $Ly6G^{high}$, and $Ly6G^{low}$ cells. Detectable CEBPβ isoforms are LAP* = 38 KD and LAP = 34 KD, as indicated. Actin was used as a loading control. Representative blot from four independent experiments.

indicative of their immature state as well as on their *in vitro* suppressive activity [15]. However, this issue has not been addressed in this study and warrants further investigation.

Thus far, MDSCs have been extensively studied in patients with different types of tumors, but rarely examined in patients with autoimmune diseases. In human IBD, MDSCs are even less characterized. Haile et al. found that mononuclear $HLA\text{-}DR^{-/low}CD14^{+}$ cells were elevated in IBD patients compared with healthy controls, while coculturing of these cells *in vitro* with autologous stimulated PBMCs resulted in a dose dependent suppression of proliferation and cytokine production [33]. Subsequently, Xi et al. confirmed the elevation of $HLA\text{-}DR^{-/low}CD14^{+}$ cells in periphery of IBD patients, but also associated the exacerbation of IBD with higher levels of MDSCs [44]. In our study, we demonstrate that MDSCs were significantly enriched in the periphery of IBD patients, and importantly, they not only failed to suppress but rather promoted responding autologous stimulated T cell

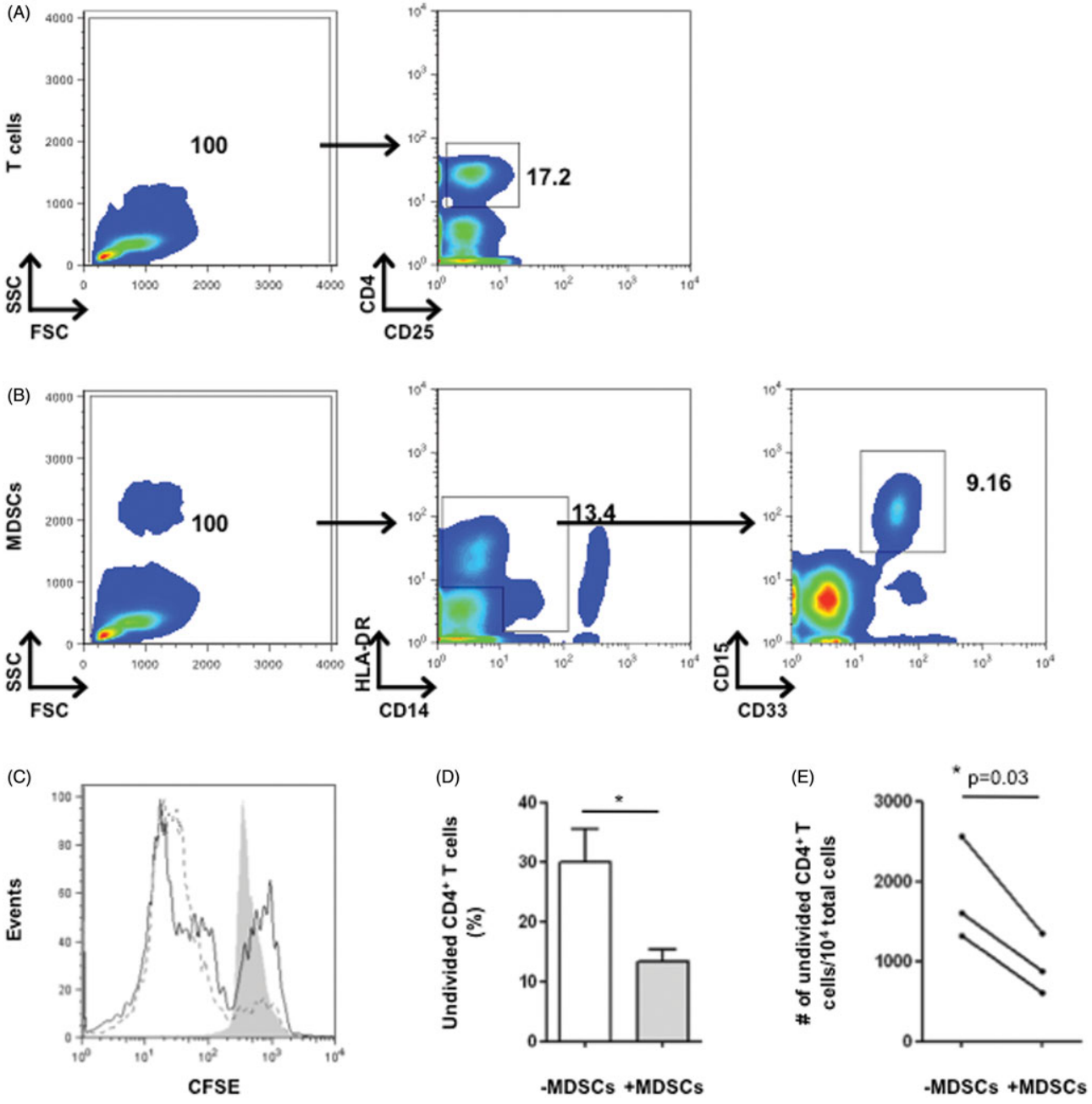

Figure 7. Human MDSCs promote T cell proliferation in IBD patients. FACS plots demonstrate the gating strategy used to isolate T cells (A) and MDSCs (B) from peripheral blood of IBD patients. (C) Sorted $CD33^+CD15^+$MDSCs from IBD patients with active disease were cocultured with autologous $CD4^+CD25^-$ T cells stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) at 1:1 ratio for 5 d. T cell proliferation was measured as CFSE dilution by flow cytometry. $CD4^+$T cells alone (grey fill), anti-CD3/CD28 activated $CD4^+$T cells (solid line), anti-CD3/CD28 activated $CD4^+$T cells plus $CD33^+CD15^+$MDSCs (dashed line). CFSE dilution graph is representative of three independent experiments. (D) The percentage and (E) total numbers of undivided $CD4^+CD25^-$ T cells in the presence or absence of MDSCs in the culture is shown. Paired *T* test *p* values: *, $p < 0.05$.

proliferation. However, in contrast to the above studies, our results refer to polymorphonuclear MDSCs defined as $HLA\text{-}DR^{-/low}CD14^-CD33^+CD15^+$cells, possibly indicating that the two subsets exert different functionality in autoimmune responses.

In experimental IBD, MDSCs were first described in a $CD8^+$T cell-mediated model of colitis. The frequency of $CD11b^+Gr1^+$MDSCs with immunosuppressive function was increased in the spleen and intestine of colitic mice. Furthermore, cotransfer of transgenic T cells with MDSCs ameliorated intestinal inflammation [33]. More recently, murine data were confirmed in chronic DSS, as well as, in TNBS-induced colitis. An increase of MDSCs in the spleen and/or intestine correlated with the severity of intestinal inflammation, while adoptive transfer of either splenic or *in vitro* generated MDSCs ameliorated enterocolitis [32–34,45]. Consistent with the above studies, our data support an increase of $CD11b^+Gr1^+$MDSCs, and more specifically $Ly6G^+$subset, in the spleen of TNBS-treated mice. However, their percentage in colon LPMCs during active TNBS-induced inflammation was rather decreased. More importantly, adoptive transfer of *in vitro* generated

$CD11b^+Gr1^+$MDSCs in TNBS-treated mice exacerbated colitic phenotype. Although, surprising at first sight, this result is in line with studies supporting a proinflammatory role of MDSCs in experimental colitis, as they differentiate into inflammatory dendritic cells and macrophages [35,37] or functioned as APC cells, triggering T cell proliferation [36]. The disparity in these results could be attributed to the functional plasticity of the heterogenous population of MDSCs, which may be dictated by the inflammatory environment and the disease stage. In support of this, it is worth mentioning here the difference between the animal models used to mimic IBD and explore MDSCs efficacy. Guan et al. [32] used a more chronic model of colitis with administration of TNBS twice over 1-week interval. By contrast, using an acute model in our study, we speculate that T cells, assumed to be the primary target of MDSCs, may not be fully engaged to suppressive signals by MDSCs.

Several lines of evidence have supported that MDSCs exert their suppressive function using a number of mechanisms. Recent data suggested that the molecular program underlying the immunoregulatory properties of MDSCs depends on CEBPβ. Marigo et al. [22] showed that lack of CEBPβ abrogated the suppressive capacity of both murine and human *in vitro* generated BM-MDSCs, at least through the reduction in arginase 1 and nitric oxide 2 proteins, while it did not affect their ability to proliferate. Consistent with this study, our results demonstrated that CEBPβ was barely detectable in freshly isolated $Gr1^+CD11b^+$, $CD11b^+Ly6G^{high}$ or $CD11b^+Ly6G^{low}$ BM cells, lacking inhibitory function. Notably, none of these cell populations isolated from TNBS-treated mice expressed CEBPβ isoforms. This finding may provide an explanation why MDSCs, although accumulated in the colitic inflammatory milieu, failed to effectively control aberrant T cell responses. Interestingly, further evidence support that CEBPβ expression in myeloid progenitor cells is directly regulated by STAT3 [46]. Therefore, it will be important to study STAT3 expression in BM-MDSCs exposed to colitic environment.

Taken together, our results indicate that *in vitro* generated BM-MDSCs bear suppressive capacity *in vitro* and *in vivo* under steady state, but fail to control colitis *in vivo*. Their intrinsic plasticity renders them prone to *in vivo* conversion into effector cells and consequent loss of their suppressive potential under inflammatory conditions. To allow application of MDSCs as immunotherapy, we could envision them as maintenance therapy to prevent disease reactivation following induction of remission with other therapeutic factors.

Acknowledgements

The authors thank M. Ioannou and C. Choulaki, for assisting with experiments; Z. Vlata, N. Gounalaki, and T. Makatounakis for technical assistance with cell sorting, E. Choustoulaki for patient care, M. Tzardi for histology preparations, and I. Drygiannakis for review of the manuscript.

Disclosures

The authors report no conflicts of interest. This work was supported by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program ''Education and Lifelong Learning'' of the National Strategic Reference Framework (NSRF) – Research Funding Program: Heraclitus II 3396. Investing in knowledge society through the European Social Fund, the Greek General Secretariat of Research and Technology [Aristeia II 3468 to P.V.] and European Union project Innovative Medicine Initiative 6 [''BeTheCure'', Contract no. 115142-2, to P.V. and D.B.].

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